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Proteinase-Activated Receptor Agonists in Regulation of Platelet Activation and Platelet-Leukocyte Interactions: Roles of Matrix Metalloproteinases

by

Ada Wing-Yee Chung ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

Department of *Pharmacology*

Edmonton, Alberta

Fall 2003

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Proteinase-Activated Receptor Agonists in Regulation of Platelet Activation and Platelet-Leukocyte Interactions: Roles of Matrix Metalloproteinases** submitted by **Ada Wing-Yee Chung** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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June 20, 2003

Dedication

Graduation is a memorable experience in one's life. I would like to share this memory and excitement with my husband, my newborn daughter and my sister. Elliott, although you don't like me spending too much time on experiments, I know that you always support my decisions and what I am doing. Thank you for your time on reading my manuscripts and thesis, and improving my writing skill. Sharon, you are the only person fully understand the frustration and difficulty in preparing my thesis, since you have been with me for the last nine months while I was writing this thesis. Wing, the long conversation with you every night has helped me release a lot of pressure and worry. Finally, I want to take this opportunity to praise my Lord, for He gives me the perseverance and knowledge to pursue in research and study.

Abstract

In response to vascular injury, platelets rapidly adhere to subendothelium via the glycoprotein (GP) Ib/IX/V receptor. Adhesion is followed by the release of ADP, matrix metalloproteinase-2 (MMP-2) and thromboxane A_2 (TXA_2), and the upregulation of GPIIb/IIIa, leading to irreversible aggregation. During platelet activation, the presence of P-selectin and microparticles mediate platelet-leukocyte interactions, facilitating leukocyte activation and an inflammatory response. In this thesis, I have studied human platelet aggregation and platelet-leukocyte interaction during stimulation of platelets and leukocytes with proteinase-activated receptor (PAR) agonists including thrombin, thrombin receptor activating peptide (TRAP) and selective PAR1- and PAR4-activating peptides. I also investigated platelet activation and aggregation, as well as leukocyte activation and platelet-leukocyte aggregation in blood from patients subjected to the cardiopulmonary bypass (CPB) procedure.

I have demonstrated that all PAR agonists studied induced platelet aggregation. Thrombin was the most potent agonist, followed by other PAR-activating peptides. PAR1- and PAR4-activating peptides synergized to induce maximal aggregation. Prostacyclin (PGI_2) and nitric oxide (NO)-donor (S-nitrosoglutathione, GSNO) reduced PAR agonist-mediated platelet responses. PAR agonist-induced aggregation was aspirin-insensitive indicating a minor role of TXA_2. Phenanthroline and apyrase inhibited thrombin- and PAR1-mediated aggregation suggesting the involvement of ADP and MMP-2 in this reaction. PAR4-mediated aggregation; however, was entirely ADP-dependent. PAR agonists induced platelet-leukocyte aggregation, P-selectin expression and microparticle generation. Prostacyclin, GSNO, aspirin, apyrase and phenanthroline attenuated platelet-leukocyte interaction, with phenanthroline being the most effective inhibitor. The surface expression and the activities of MMP-1, -2, -3 and –9 were upregulated during PAR stimulation. Platelet-leukocyte aggregation was attenuated by monoclonal antibodies against MMP-1, -2 and –3, while antibodies against MMP-9 resulted in a biphasic regulation (stimulation at lower and inhibition at higher concentrations) of aggregation. During the CPB procedure, both platelet activation and platelet-leukocyte aggregation were seen, and these were accompanied by a depressed aggregatory response. Iloprost (PGI_2 analogue), NO, or the combination of NO and iloprost, prevented platelet activation and preserved platelet function. These results underlie a crucial role of thrombin, PAR agonists and MMPs in platelet functions and platelet-leukocyte interactions.

Acknowledgements

I would like to take this precious opportunity to say thank you to numbers of people helping me throughout my PhD studies. My supervisor, Dr Marek Radomski, has encouraged me to pursue in research and helped me to develop self-confidence. I appreciate the friendship with Dr Anna Radomski, who has offered me various support and opinion in both experimental and personal issues. My colleagues including Macelo Marcet, Drs Paul Jurazs and David Alonso, I thank for their friendship and valuable suggestions in my experiments. Definitely, it is a memorable time working in this lab with them.

Being the first person introducing me the concept of platelet-leukocyte interaction, Dr Michael Stewart also offered assistantship in the phase-contrast microscopy and flow cytometry. I also want to thank the rest of my committee, Drs Alexander (Sandy) Clanachan, Peter Light and Esmond Sanders, for their helpful suggestions during each committee meetings and in the preparation of my thesis.

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List of Abbreviations

ASA	acetylsalicylic acid (aspirin)
CABG	coronary artery bypass grafting
CPB	cardiopulmonary bypass
FITC	fluorescein-isothiocyanate
GP	glycoprotein
GSNO	S-nitrosoglutathione
ICAM	intercellular adhesion molecule
LEA-1	lymphocyte function-associated antigen-1
Mac-1	macrophage-1 antigen
MFI	mean fluorescence intensity
MMP	matrix metalloproteinase
MoAbs	monoclonal antibodies
MP	microparticle
NO	nitric oxide
ODQ	1H-[1,2,4]Oxadiazole[4,3]quinoxalin-1-one
PAR-AP	proteinase-activated receptor-activating peptide
PE	phycoerythrin
PGI_2	prostacyclin
PLA	platelet-leukocyte aggregate
PSGL-1	P-selectin glycoprotein ligand-1
RGD	Arg-Gly-Asp
SEM	standard error mean
TIMP	tissue inhibitor of matrix metalloproteinase
TRAP	thrombin receptor activating peptide
TXA_2	thromboxane A_2
WBC	white blood cell
vWf	von Willebrand factor

Introduction

I. Hemostasis and Thrombosis

Hemostasis is the combined effect of various homeostatic mechanisms involved in the prevention of spontaneous hemorrhage and in arresting blood loss during vascular injury. In contrast, uncontrolled formation of the hemostatic plug (thrombus) in the circulation results in life-threatening *thrombosis*. Effective hemostasis depends on normal function of its vascular, platelet, coagulation and fibrinolytic components.

Vascular Component

As early as in 1856, Virchow stressed the fundamental role of the vessel wall in the hemostatic process and in the formation of thrombi. The immediate control of bleeding from an injured vessel is accomplished by a transient vasoconstriction. In general, the vascular component of hemostasis can be anatomically and functionally subdivided into the vascular supporting tissue, the blood vessel wall, and the vascular endothelium. Integrity of the supporting tissue and vessel wall are vital for the prevention of hemorrhage, while integrity of the vascular endothelium is important primarily for the prevention of thrombosis.

The hemostatic response to endothelial damage is contingent upon the coordinated and reciprocal interactions between platelets and vessel wall constituents (Body, 1996). Under basal conditions, platelets circulate in the resting state and do not adhere to the endothelium. The endothelium maintains this non-adherent and non-thrombogenic surface by synthesizing releasable mediators such as prostacyclin (PGI_2) and nitric oxide (NO), both of which inhibit platelet activation by stimulating adenylyl cyclase and guanylyl cyclase, respectively, and thereby augmenting levels of cAMP and cGMP (Best *et al*, 1977; Gerzer *et al*, 1988). Endothelin, a potent vasoconstrictor, enhances these mechanisms by stimulating PGI_2 synthetase and NO synthase (Herman *et al*, 1989; Lidbury *et al*, 1990). Recent findings have shown that endothelial cells have the capacity to generate matrix metalloproteinase-9 (MMP-9) (Zucker *et al*, 1995), another inhibitor of platelet activation (Fernandez-Patron *et al*, 1999a). Endothelial cells also constitutively express thrombomodulin, a surface receptor that binds thrombin and facilitates the activation of the coagulation inhibitor protein C; and ADPase, which converts platelet agonist ADP to AMP (Leopold & Loscalzo, 1995). Therefore, endothelial dysfunction perturbs the homeostatic mechanisms and promotes platelet adhesion and activation (Griendling & Alexander, 1996) (Figure 1).

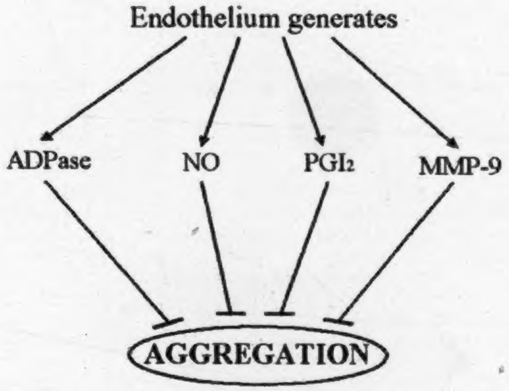


Figure 1. Inhibitor pathways of platelet function. Normal endothelium generates at least four inhibitors, ADPase, NO, PGI_2 and MMP-9 to suppress platelet aggregation.

Formation of Platelet Plug, Platelet Physiology and Biochemistry

In a normal hemostatic process, platelets play a critical role in the recognition of injured vasculature, formation of hemostatic plugs, prevention of bleeding and wound healing. Platelets are multipotent cells both with respect to the number of agonists and number of responses. The basic platelet responses include adhesion, shape change, secretion, and aggregation. Understanding these reactions requires basic knowledge of platelet morphology, physiology and biochemistry (Figure 2) (Rao, 1999).

Platelets originate from the fragmentation of megakaryocyte cytoplasm. They are discoid about 2 μm in diameter and 1 μm thick and as such are the smallest blood cells. Platelets circulate in the blood stream as anucleate cells, and have a lifespan of 14 days in human. Normal blood contains from 150,000 to 450,000 platelets per μL. The plasma membrane consists of phospholipids that are arranged in such way that the negatively charged phosphatidylinositols and phosphatidylserine are located at the inner leaflet of the membrane. These lipids are involved in cellular signaling. The plasma membrane contains a number of disulfide-bonded glycoproteins (GPs), some of which belong to the integrin family, a widely distributed family of α: β heterodimer complexes that function as receptors for adhesive proteins (Ginsberg *et al*, 1993). Filamentous structures that are localized beneath the cell membrane provide support for membrane and are endowed with contractile properties important for platelet shape change, contraction and secretion. The cytoskeleton in platelets consists primarily of actin filaments. Other contractile proteins include myosin, tropomyosin, actin, α-actinin and gelsolin (Fox, 1993). Platelets have most of the subcellular organelles that are found in other cells except for the nucleus; therefore, they are deficient in DNA, except for mitochondrial DNA. There are numerous organelles in the cytoplasm: mitochondria, peroxisomes, lysosomes, glycogen and storage granules, where α-granules and dense granules are platelet-specific and are released to the extracellular milieu upon platelet activation. Platelets contain two different intracellular membrane systems. The dense tubular system is a closed system, which is the site for calcium storage and prostaglandin metabolism (Gerrard *et al*, 1978). The open canalicular system, the invagination of the plasma membrane, provides an increase in surface area for the uptake and release of secretory products from the storage granules. Many platelet receptors mediate their responses through heterotrimeric GTP-binding proteins (G proteins), which function as transducers between the seven-transmembrane domain receptors and the intracellular effector enzymes {i.e. phospholipase A_2 (PLA_2), phospholipase C (PLC) and adenylyl cyclase}(Offermanns, 2000). Agonist-mediated platelet activation stimulates PLC, which hydrolyzes phosphatidylinositols 4,5-bisphosphate to form second messengers such as diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Majerus, 1992). In the presence of appropriate phospholipids, DAG activates protein kinase C (PKC), resulting in phosphorylation of various intracellular proteins. Protein kinase C activation is crucial for platelet secretion and for the expression of platelet surface fibrinogen binding sites, which is a prerequisite for platelet aggregation (Nishizuka, 1986).

IP_3 mobilizes calcium from the dense tubular system. Elevated cytosolic calcium, either through the release from intracellular stores or the influx from extracellular milieu, is essential for most platelet functions and is considered as an important regulator in platelet physiology (Berridge, 1993). One of the examples of calcium-dependent process in platelets is the release of arachidonic acid from phospholipids by the action of calcium-dependent PLA_2; this is the rate-limiting step in thromboxane A_2 (TXA_2) synthesis. The free arachidonic acid, generated by the hydrolysis of phospholipids by PLA_2, is converted by cyclooxygenase to cyclic endoperoxides such as prostaglandins G_2 and H_2, and subsequently by thromboxane synthetase to TXA_2 (Siess, 1989). TXA_2 is a major metabolite of arachidonic acid in platelets. It functions both as a vasoconstrictor and a potent platelet agonist. In endothelial cells where prostacyclin synthetase is present, arachidonic acid-synthesized cyclic endoperoxides are converted to PGI_2, which is a vasodilator and a potent platelet inhibitor. Acetylsalicylic acid (aspirin, ASA) suppresses platelet secretion by inhibiting cyclooxygenase in platelets and in vessel walls. This action results in reduced synthesis of TXA_2 in platelets and PGI_2 in the vessel wall. Cyclooxygenase, in platelets, is acetylated and inhibited by lower concentrations of ASA than that in the vessel wall. Moreover, since platelets lack the capacity for *de novo* protein synthesis, the inhibitory effects of ASA persist for the time necessary for the production of new platelets from megakaryocytes to take place. In contrast, in the endothelium, the ASA-inactivated cyclooxygenase can be replaced by the *de novo* synthesized enzyme approximately 2 days after administration of ASA. Therefore, ASA causes strong inhibition of TXA_2 formation in platelets, modest reduction of PGI_2 in endothelial cells and overall inhibition of platelet aggregation that persists throughout the platelet life span of 8-11 days (Dusting *et al*, 1982).

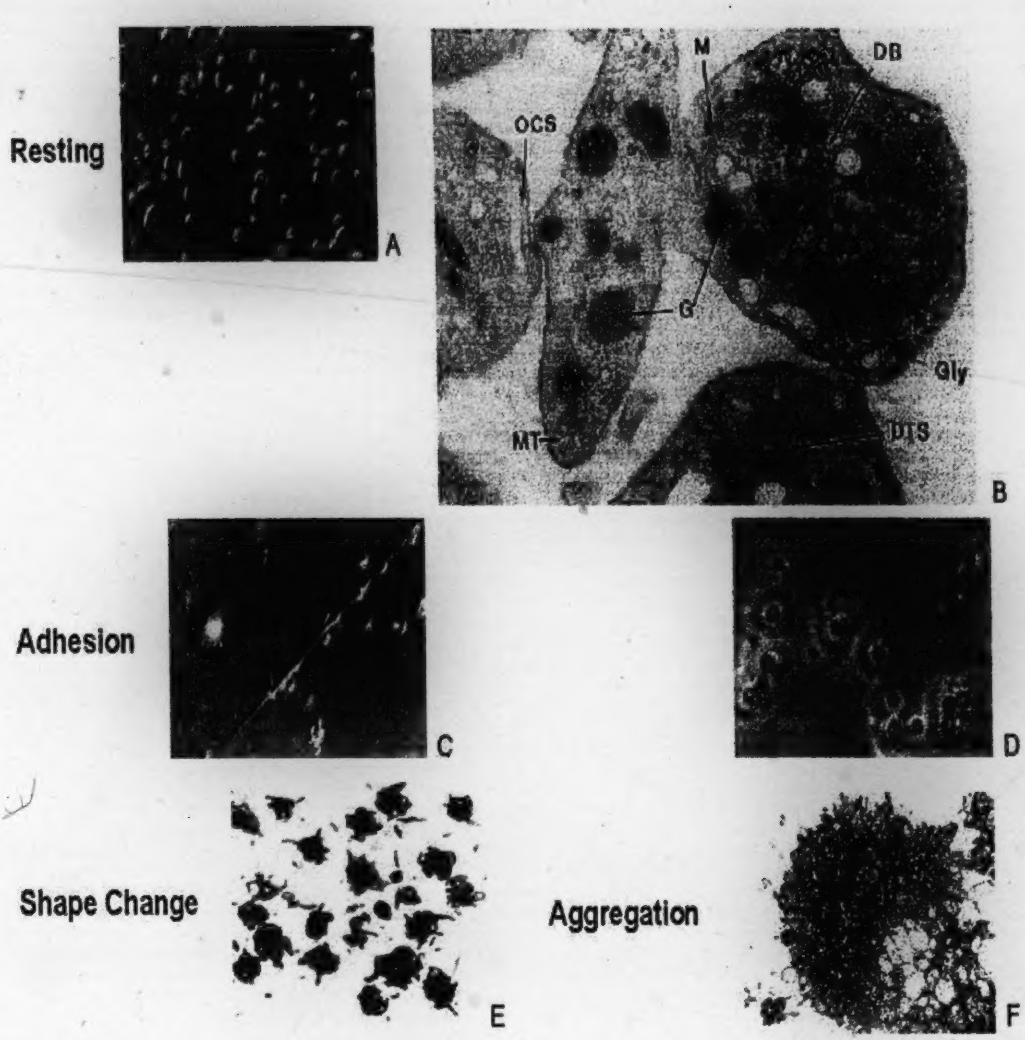

Resting
A
M
DB
OCS
G
Gly
DTS
MT
B
Adhesion
C
D
Shape Change
E
Aggregation
F

Figure 2. Platelet Physiology. Resting platelets observed by phase contrast microscopy (A, x800) and transmission electron microscopy (TEM; B, x40000). They have a characteristic disc-like appearance. Organelles such as α-granules (G), microtubule (MT), dense bodies (DB), open canalicular system (OCS), dense tubular system (DTS), mitochondria (M) and glycogen (Gly) can be seen with TEM. Platelet adhesion on collagen fibers (C) and vWf-coated beads (D). Platelet activation resulting in shape change with the formation of pseudopodia (E, x6500) and the subsequent aggregation (F, x40000) (adapted from Read *et al*, 1985).

Adhesion

Platelet adhesion to the vascular wall is tightly regulated in order to maintain both the fluid state of blood and the ability to promptly form a hemostatic plug at sites of vascular injury. Platelets do not adhere to intact endothelium, but following vessel injury, subendothelial components such as collagen and von Willebrand factor (vWf) are exposed and platelets have a strong affinity to these proteins. Although fibrillar collagens, particularly types I and III, are important structural components of the extracellular matrix and can directly bind and activate platelets via receptors such as GPIa/IIa and GPIV, platelet adhesion to exposed collagen in the subendothelium is largely mediated by vWf (Alberio & Dale, 1999).

Von Willebrand factor is synthesized in megakaryocytes and endothelial cells as a prepro molecule, and is also found in platelet α-granules and subendothelial matrix. The prepro vWf is composed of signal peptide, propolypeptide and mature subunit. The domain structure consists of repeated A, B, C and D domains, which provides the potential for multiple contact sites with platelets and collagen. A1 and A3 domains are recognized by GPIb and collagen type I and III; C1 domain contains the Arg-Gly-Asp (RGD) sequence recognized by GPIIb/IIIa complex (Berndt *et al*, 1995).

Initial adhesion of platelets to the subendothelium is mediated mainly through the interaction of matrix-bound vWf with the platelet surface receptor GPIb/IX/V complex. The GPIb/IX/V complex contains four polypeptides: GPIbα and GPIbβ linked covalently by the disulphide bond are originally identified as GPIb. GPIX and GPV are associated with this complex noncovalently. The GPIbα subunit is essential for binding vWf, while both GPIbβ and GPIX are important in assembling and anchoring the complex to the platelet surface (Kenny *et al*, 1999). GPV is shown to be non-essential for complex assembly or function and its main role is suggested to be a negative regulator of thrombin action on platelets (Ramakrishnan *et al*, 1999). In platelets, GPIb complex is associated with cytoskeletal components. This association is important in GPIb-mediated signal transduction (Solum & Olsen, 1984). Using monoclonal antibodies Berndt and colleagues (1985) found that there are 25000 copies of GPIbα on the cell surface. The study found also approximately the same number of GPIX molecules on the cell surface and suggested that these components are synthesized in a coordinated manner.

Von Willebrand factor-GPIb-mediated platelet adhesion becomes highly important at higher shear forces. Indeed, patients with Bernard-Soulier syndrome, whose platelets lack the functional GPIb/IX/V complex, show impaired platelet adhesion to subendothelium at high shear rates (Weiss *et al*, 1978). Shear forces are generated by the differences in velocity of adjacent blood elements as they approach the vessel wall. Shear rates in the human body vary from 50 to 3000 s^{-1}. Interestingly, shear forces are significantly higher at vascular branching and in pathologically stenosed vessels (Slack *et al*, 1993). At lower shear rates, more abundant matrix proteins such as fibronectin mediate platelet adhesion; however, this adhesion is readily reversed by increasing shear (Sixma & Groot, 1991). High shear stress promotes vWf-GPIb binding by inducing a conformational change in vWf, thereby exposing a cryptic binding site for GPIb (Ikeda *et al*, 1991). Therefore, platelet adhesion at high shear is exclusively vWf-dependent. This is thought to be a selection effect, since high shear disrupts most receptor-ligand interactions except the shear-resistant binding of vWf to GPIb.

In many experiments, inhibition of the vWf-GPIb interaction does not completely abolish platelet adhesion. This leads to the recognition that vWf also binds to platelet GPIIb/IIIa via the RGD sequence (Weiss *et al*, 1989). In fact, both vWf receptors may be required for maximal platelet binding, but the relative contribution of GPIb/IX/V and GPIIb/IIIa may vary depending on the substrate (Fressinaud *et al*, 1988; Danton *et al*, 1994). It is generally agreed that GPIb is the primary receptor for matrix-associated vWf with GPIIb/IIIa binding occurring secondarily. The shear-resistant GPIb/vWf interactions capture and slow down platelets, allowing GPIIb/IIIa to become activated and mediate permanent platelet adhesion and eventually platelet aggregation.

Shape Change

Platelet shape change from discoid to amoeboid is mediated by the circumferential band of microtubuli and it is characterized morphologically by the extension of pseudopodia (Rao, 1999). The mechanisms involve changes in the arrangement of cytoskeletal proteins and phosphorylation of myosin light chain. During shape change, platelet granules fuse with the membrane of open canalicular system, and are ready to be released (Stenberg *et al*, 1984).

Secretion

Platelets contain three different types of secretory granules: α-granules, dense granules and lysosomes (Table 1). Following platelet stimulation, the contents of these granules are extruded from the platelet interior in a process involving the fusion of the granules and the surface-connected open canalicular system (Holme *et al*, 1974). The granules differ from each other by the strength of aggregating stimuli that are necessary to induce secretion. Lysosomal secretion occurs only after stimulation with strong agonists (i.e. thrombin and collagen) and this release is slow and is never completed. Weak agonists such as ADP and adrenaline induce the dense and α-granule secretion and these releases are fast and almost complete (Verhoeven *et al*, 1984).

Table 1. Contents of storage granules in platelets

α-granules	Dense granules	Lysosomes
Fibrinogen	ATP, GDP	β-galactosedase
Fibronectin	GTP, GDP	β-glucuronidase
vWf	calcium	β-hexosaminidase
Vitronectin	Serotonin	
Factors Va and VIII		
PDGF		
TGF-β		
Thrombospondin		
Albumin		
Platelet factor 4		
P-selectin		

Recent studies have shown that the members of MMPs (i.e. MMP-1, 2, 3 and 9) are released from cytoplasm during platelet activation. MMPs comprise an ever-growing family of zinc-dependent endopeptidases, exhibiting differential proteolytic activity against extracellular matrix proteins (Parsons *et al*, 1997). More than twenty MMPs have been identified and sequenced to date, and they are given both a descriptive name (for example, interstitial collagenase is found in the interstitial space and specifically degrades fibrillar collagen) and an MMP number in the order of their identification. Based on substrate preferences, MMPs are divided into subgroups: collagenase, gelatinase, stromelysin and a group including macrophage elastase. In addition, there are at least four membrane-type (MT)-MMPs that are characterized by their association with the plasma membrane. All MMPs have a similar domain structure as shown in Figure 3. Signal peptide, serving as a signal for transporting the protein into the endoplasmic reticulum, is lost after being exported from the cell. Within the propeptide domain, the cysteine (cys) (the 'Cys switch') coordinates with the catalytic zinc (Zn) atom, and maintains the latency of pro-MMPs. Catalytic domain consists of a highly conserved Zn-binding active site. There are also additional binding sites for calcium ions and the structural Zn atom, which are required for the stability and the expression of enzymatic activities. MMP-2 and MMP-9 have three repeats of the fibronectin type II domain inserted ahead of the catalytic Zn-binding region. These inserts assist in binding of gelatinases to their substrates. Between the catalytic domain and the hemopexin-like domain is a proline-rich region ('hinge region'), which permits the C-terminal domain to fold back on the catalytic domain. The hemopexin-like domain contains four repeats that resemble hemopexin and vitronectin. This region is required for the collagenase cleaving triple helical interstitial collagens, the cell surface activation of pro-MMP-2 by MT-MMP, and the binding of tissue inhibitors of metalloproteinases (TIMPs). In contrast to most MMPs having their C-terminus at the end of the hemopexin domain, MT-MMPs have a transmembrane domain that anchors the enzymes into the plasma membrane. The hemopexin domain is absent in the smallest MMP, matrilysin (MMP-7) (Nagase & Woessner, 1999). The expression, activation and activity of MMPs are tightly controlled. Tissue inhibitors of metalloproteinases 1-4 are specific endogenous inhibitors of MMPs that regulate the biological activity of these proteinases (Vincenti *et al*, 2001). MMP-1 and MMP-2 stimulate platelet adhesion and aggregation (Sawicki *et al*, 1997; Galt *et al*, 2002; Jurasz *et al*, 2002). MMP-9 is expressed in platelets in lower amounts than MMP-1 or MMP-2. This MMP counteracts the platelet-aggregatory effects of MMP-2 by inhibiting aggregation, while MMP-3 is devoid of any effects on aggregation (Fernandez-Patron *et al*, 1999a; Galt *et al*, 2002). In resting platelets, these enzymes are stored in the latent form. In contrast to leukocytes where pro-MMP-2 and pro-MMP-9 are stored in specialized gelatinase granules (Tschesche, 1997; Opdenakker *et al*, 2001), in platelets, pro-MMP-2 is found in the cytoplasm without an apparent association with α or dense granules (Sawicki *et al*, 1998). In human platelets, TIMP-4 colocalizes with MMP-2 in platelet cytoplasm, is released upon secretion and acts as the major inhibitor regulating the activity of MMP-2 in platelets (Radomski *et al*, 2002).

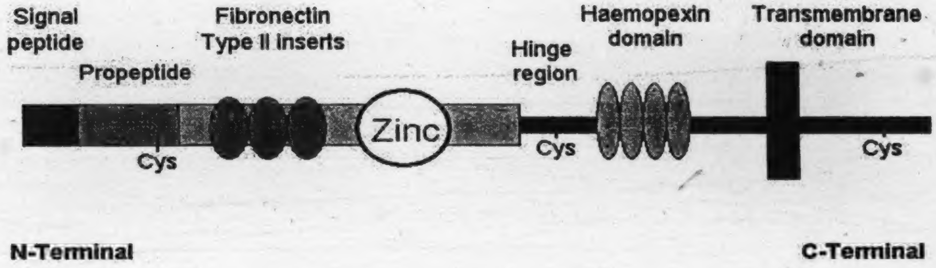

Signal peptide
Propeptide
Fibronectin Type II inserts
Hinge region
Haemopexin domain
Transmembrane domain
Zinc
Cys
Cys
Cys
N-Terminal
C-Terminal

Figure 3. General structure of matrix metalloproteinases (MMPs) (adapted from Nagase & Woessner, 1999).

During secretion, granule membranes are translocated to the surface membrane via the open canalicular system. Specific membrane glycoproteins (originally facing the interior of the granules) are carried with granules to the surface membrane and they appear there as neoepitopes. They include activated fibrinogen-receptor (GPIIb/IIIa), P-selectin (also called GMP140, PADGEM and CD62) from α-granule membrane, granulophysin from dense granule membrane, lysosome-associated membrane protein and CD63 released from lysosomes (Stenberg *et al*, 1985; Berman *et al*, 1986; Israels *et al*, 1992). All these factors can be used as markers of platelet activation.

Contents of the α-granules (such as thrombospondin, platelet factor 4, fibrinogen, vWf, fibronectin and platelet-derived growth factor) and dense granules (such as ADP and serotonin) can bind to their respective receptors on the platelet surface. The interactions between these mediators and their receptors trigger intracellular responses and regulate platelet functions. Therefore, secretion is considered to be a positive-feedback mechanism to promote irreversible platelet aggregation and thrombus growth by autostimulating platelets and recruiting additional platelets to the sites of injury. There are at least three mediators released during platelet activation, ADP, TXA_2 and MMP-2. These mediators trigger transduction mechanism reactions (pathways) that lead to aggregation. Pharmacological inhibitors of their cellular effects are used to evaluate the role of each mediator in aggregation (Sawicki *et al*, 1997; Jurasz *et al*, 2001; Radomski *et al*, 2001) (Figure 4).

Aggregation

The combined effects of ADP, TXA_2 and MMP-2 lead to the up-regulation of the major platelet surface receptor, the GPIIb/IIIa complex (Shattil *et al*, 1998). As evidenced in

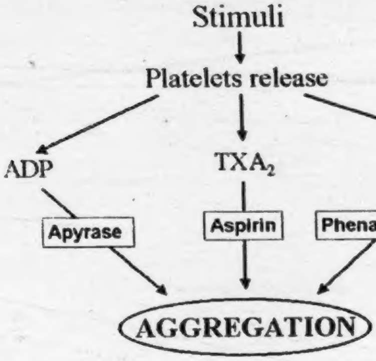


Figure 4. Activator pathways of platelet aggregation. Activated platelets release at least three major mediators, ADP, TXA_2 and MMP-2 to potentiate platelet aggregation. Apyrase, aspirin and phenanthroline are relatively selective inhibitors of ADP-, TXA_2- and MMP-2-dependent pathways, respectively.

patients with Glanzmann's thrombasthenia, a deficiency of functional GPIIb/IIIa receptors on platelets results in impaired adhesion and aggregation. The GPIIb/IIIa complex is the most abundant platelet surface receptor. Approximately 80000 copies of GPIIb/IIIa are randomly distributed on the plasma membrane, open canalicular system membrane, and the membrane of α-granules. It is a calcium-dependent heterodimer complex of membrane glycoproteins, GPIIb (integrin α_{IIb} subunit) and GPIIIa (integrin β_3 subunit) (Brass *et al*, 1985). The α_{IIb} subunit appears to be platelet- and megakaryocyte-specific, and forms a complex with β_3 subunit. The α_{IIb} consists of a heavy chain (GPIIbα) and a light chain (GPIIbβ) linked by a disulfide bond, whereas β_3

is a single chain glycosylated polypeptide. Ligand binding to GPIIb/IIIa requires divalent cations, which are necessary not only for the formation of a functional receptor complex, but also directly participate in the formation of ligand recognition sites (Shattil *et al*, 1986).

The main role of the GPIIb/IIIa complex is to serve as a receptor for a number of adhesive proteins that mediate platelet adhesion and aggregation. The first identified ligand was the abundant plasma coagulation factor, fibrinogen. Plasma fibrinogen, which is originally synthesized by hepatocytes but not in megakaryocytes, is packaged into the α-granules of megakaryocytes and platelets (Du, 2000). Fibrinogen is a soluble protein with a molecular weight of 340 kDa that is composed of three pairs of disulfide linked α, β and γ chains. The α chain contains two RGD sequences recognized by GPIIIa, while γ chain contains a sequence at the carboxyl terminus recognized by GPIIb. Interestingly, GPIIb/IIIa can also bind other adhesive proteins including fibronectin, vWf, and vitronectin, and the binding mechanism is, in part, mediated by the RGD sequence (Phillips *et al*, 1988).

In resting platelets, GPIIb/IIIa does not mediate platelet aggregation. This is probably due to a lack of high affinity binding sites for GPIIb/IIIa ligands on the surface of unstimulated platelets. Indeed, the monoclonal antibody AP7 that contains an RGD-like recognition sequence and has high affinity to GPIIb/IIIa, interacts with these receptors without platelet activation. Platelet activation induces the surface expression and ligand-binding function of an additional pool of GPIIb/IIIa complex derived from membranes of α-granules and the open canalicular system (Woods *et al*, 1986; Sims *et al*, 1991). The activated conformation of GPIIb/IIIa is specifically recognized by the activation-dependent, ligand-mimicking antibodies (PAC-1) that are frequently used in flow cytometry to quantify platelet activation.

Two signaling pathways so called "inside-out" and "outside-in" signaling are involved in the regulation of GPIIb/IIIa ligand binding affinity. As discussed above, the GPIIb/IIIa integrin on unstimulated platelets exists in an inactive form showing low affinity for soluble ligand fibrinogen and vWf. This affinity is highly increased following stimulation of platelets by agonists such as thrombin, collagen and ADP. As a consequence of this inside-out signaling, affinity modulation of $\alpha_{IIb}\beta_3$ for fibrinogen and vWf is the primary driving force for the formation of initial platelet-platelet contacts and to initiate aggregation. This primary contact subsequently initiates the outside-in signaling involving the release of platelet mediators that can act back on platelets. This is a driving force for the consolidation and stabilization of platelet aggregates. The outside-in signaling induces diverse functions that are critical to not only platelet functions, but also other responses of the vasculature. For instance, the synthesis of TXA_2 results in vasoconstriction and the formation of microparticles, which stimulate blood coagulation (Nieuwland *et al*, 1997).

Coagulation

Blood coagulation, the conversion of fluid blood to a solid clot by the formation of insoluble fibrin strands from soluble fibrinogen, is considered as a combination of two enzymatic cascades: The *in vivo* pathway (or extrinsic pathway, which is activated by the presence of compounds not normally found in the circulation) and the contact pathway (or intrinsic pathway, which requires only circulating factors normally present in the blood) (Figure 5). At each step of the pathway, the inactive precursors are converted to active products through proteolytic cleavage. The activated enzymes then act on the next downstream factor, generating large amounts of its active form. An important aspect is that at each step the magnitude of the signal being transduced is amplified. Therefore, a very small amount of initiating signal can be rapidly converted into a very large response, as the cascade progresses (Furie & Furie, 1992).

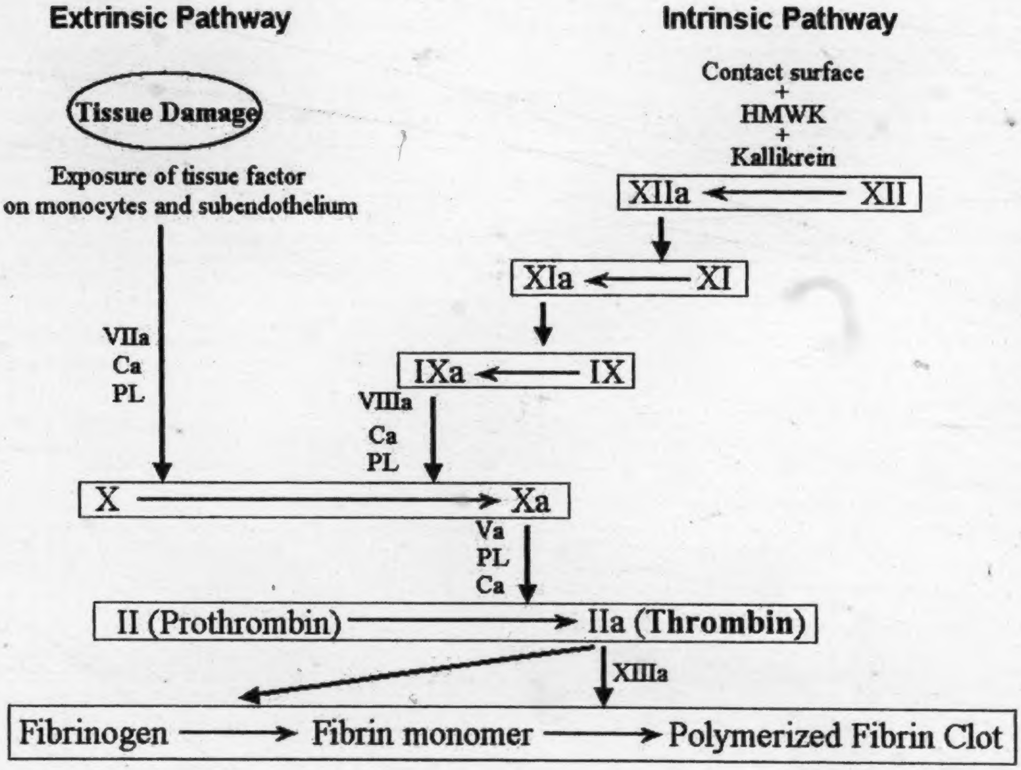

Extrinsic Pathway
Intrinsic Pathway
Tissue Damage
Exposure of tissue factor on monocytes and subendothelium
Contact surface + HMWK + Kallikrein
XIIa
XII
XIa
XI
IXa
IX
VIIa
Ca
PL
VIIIa
Ca
PL
X
Xa
Va
PL
Ca
II (Prothrombin)
IIa (Thrombin)
XIIIa
Fibrinogen
Fibrin monomer
Polymerized Fibrin Clot

Figure 5. Blood coagulation cascade. The intrinsic and extrinsic pathway merge at the level of factor X activation, and factor Xa stimulates the generation of thrombin. Primary platelet plug is therefore stabilized by the thrombin-induced fibrin linkage (PL: negatively charged phospholipid; HMWK: high molecular weight kininogen).

In the *in vivo* pathway, the major trigger is tissue factor, which is an integral membrane protein expressed on the surface of tissue cells and stimulated monocytes. It functions as a cofactor for the activation of the serine proteinase factor VII, and forms a complex with activated VII. In the presence of calcium and phospholipid, tissue factor/VIIa complex subsequently activates factor X, which then converts factors II (prothrombin) to factor IIa (thrombin). The major checkpoint in this system is the factor Xa-dependent feedback inhibition of tissue factor/VIIa complex by tissue factor pathway inhibitor. The contact pathway is initiated by the activation of factor XII (Hageman factor) in the presence of kallikrein. The active Hageman factor then gives rise to factors XIa, IXa and Xa following the activation scheme. Therefore, the two coagulation pathways merge at least at the level of factor X activation and the conversion of prothrombin to thrombin. Thrombin cleaves fibrinogen to generate fibrin monomers, which polymerize into a clot that is stabilized by a transamidase reaction catalyzed by factor XIIIa, resulting in covalent linkages between adjacent fibrin fibrils (Sidelmann *et al*, 2000).

The surface of the activated platelets is considered to be crucial for coagulation. During platelet activation, negatively charged aminophospholipids (i.e. phosphatidylserine) are exposed in the outer leaflet of the plasma membrane (so called ‘flip-flop’ process), and simultaneously platelet factor Va from α-granules is released and translocated to the surface. In the presence of calcium, the coexpression of this phospholipid and factor Va serves as a binding site for Xa. Prothrombin can then bind to this complex on the platelet surface and this proenzyme is rapidly cleaved to thrombin (Kane & Davie, 1988). This directs the fibrin formation to the site of damage and enforces the sealing effect of the platelet plug. Therefore, platelets promote blood coagulation by exposing the procoagulant surface as specific binding sites for the coagulation proteins, and protecting the coagulation enzymes from inactivation by naturally occurring plasma inhibitors.

Fibrinolysis

Fibrinolytic mechanism is vital to the prevention of disseminated clotting and the reestablishment of vascular integrity. The principal factor in this system is the proenzyme plasminogen, a serine proteinase that is converted to plasmin thereby acquiring enzymatic activity. Plasmin attacks soluble fibrinogen or fibrin and enzymatically degrades the clot, producing a number of fibrinogen degradation products. Activation of plasminogen is related to three distinct pathways. Tissue plasminogen activator, a substance derived from vascular endothelium appears to be the principal physiologic activator of plasminogen. Urokinase-type plasminogen activator, by binding to a specific cellular receptor mediates pericellular proteolysis during tissue remodelling and cell migration. In addition, factor XII or thrombin in the intrinsic pathway can activate plasminogen. The plasminogen/plasmin system can be controlled at the level of plasminogen activators by plasminogen activator inhibitor, and at the level of plasmin by α_2-antiplasmin or macroglobulin (Hong & Kwaan, 1999) (Figure 6).

In summary, hemostasis is a complex sequence of interrelated events involving the vessel wall, platelets, coagulation and fibrinolytic systems. Vascular injury is followed by reflex and humorally stimulated transient vasoconstriction. When a blood vessel is damaged, platelets exposed to subendothelial elements are stimulated to undergo a sequence of reactions leading to a primary platelet plug. Vascular contractions and primary platelet plug formation are responsible for the primary phase of hemostasis and the initial arrest of bleeding. Activation of the coagulation cascade, a sequence of enzymatic reactions resulting in fibrin formation, completes the secondary phase of hemostasis and solidifies the primary platelet plug. Fibrinolytic reactions not only limit the clot formation at the damaged area, but also promote wound healing and restore blood flow. A finely-tuned regulation entailing the cross talk among the vasculature, platelets, coagulation and fibrinolysis ensures the effectiveness of hemostasis and prevents the life-threatening thrombosis. Nature has chosen a plasma proteinase, thrombin, acting on platelets as the vital link between platelets and the coagulation system.

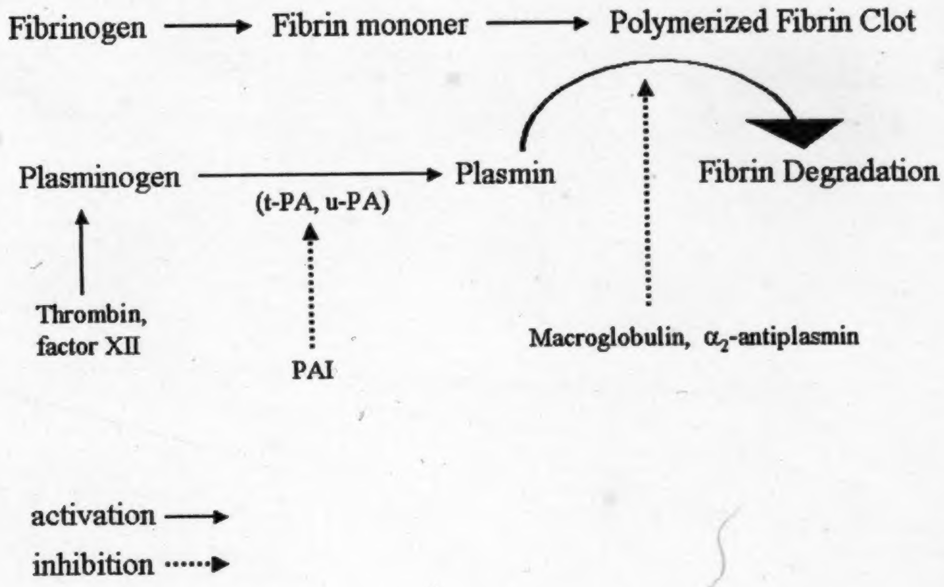

Fibrinogen
Fibrin mononer
Polymerized Fibrin Clot
Plasminogen
(t-PA, u-PA)
Plasmin
Fibrin Degradation
Thrombin, factor XII
PAI
Macroglobulin, α2-antiplasmin
activation
inhibition

Figure 6. Components and inhibitors of the plasminogen-plasmin system (t-PA: tissue-type plasminogen activator; u-PA: urokinase-type plasminogen activator; PAI: plasminogen activator inhibitors).

II. Proteinases

Platelets, leukocytes and endothelial cells exist in a proteinase-filled environment, particularly following tissue injury or inflammation. One such proteinase is thrombin, but others also exist including coagulant enzymes, protein C, plasmin and urokinase. Proteinases such as cathepsin G, elastase and tryptase can also be secreted locally by leukocytes and mast cells. Understanding the roles and mechanisms of actions of proteinases is beneficial for investigating the possible cross talk among each cell types in the vasculature.

Classification of Proteinases

Proteinases are, by definition, enzymes that hydrolyze the bond between amino acid residues in the peptide linkage of proteins. Four categories of proteinases have been identified in human tissue: serine, cysteine, aspartic and metallo-proteinases. The classification of proteinases is based on the specific amino acid residue at the catalytic site (Table 2).

Table 2. Common proteinases present in human tissues

Family	Representative Proteinases
Serine Proteinase	Thrombin, Chymotrypsin, Subtilisin, Trypsin, Elastase, Pancreatic kallikrein
Cysteine Proteinase	Papain, Cathepsins B, H, L, Caspases
Aspartic Proteinase	Penicillopepsin, Pepsin, Renin
Metalloproteinase	Carboxypeptidase I, Thermolysin, Matrix metalloproteinases (MMPs)

Thrombin Physiology

One of the most important proteinase in hemostasis and vascular biology is thrombin, which is a multifunctional serine proteinase generated during the stimulation of coagulation. The main event in this cascade is the conversion of soluble fibrinogen into insoluble fibrin, thus reinforcing the hemostatic plug. Thrombin also plays an important role in platelet activation and aggregation being the most potent platelet agonist *in vitro*. It initiates a wide range of platelet responses: shape change, secretion of ADP, serotonin and TXA_2, mobilization of the adhesion molecule P-selectin to the platelet surface, activation of GPIIb/IIIa complex, and the expression of procoagulant surface, which supports the generation of additional thrombin on platelet surface (Coughlin, 2000). In endothelial cells, thrombin induces the release of vWf, expression of adhesion molecules, and production of chemokines (Hattori *et al*, 1989; Henn *et al*, 1998). These effects not only further promote the coagulation process and platelet aggregation, but also facilitate the rapid adherence of leukocytes to the endothelial cell layers (Zimmerman *et al*, 1994). Thrombin also stimulates endothelial contraction and increases permeability (Bogatcheva *et al*, 2002), which along with increased adhesion molecule expression, facilitate rolling and transmigration of leukocytes to the site of vessel damage. In blood vessels, thrombin mediates endothelium-dependent vasodilation; however, in the absence of endothelium, it causes vasoconstriction. In the fibrinolytic system, the role of thrombin is described as the 'Thrombin Paradox' (Griffin *et al*, 1995), since the mechanism of actions of thrombin is dependent on its concentration. Low levels of thrombin binding to thrombomodulin lose its coagulant activity and gain the ability to activate protein C, which inhibits coagulation factors Va and VIIIa and downregulates coagulation process. At high concentrations, thrombin not only overcomes the anticoagulant effect of protein C, but stimulates the thrombin activatable fibrinolysis inhibitor, which inhibits plasminogen activation thereby limiting the fibrinolytic process (Esmon, 2001).

Thrombin is the key enzyme involved in hemostasis playing crucial roles at all levels of this process. Many of its cellular effects are consistent with a primary role in vessel wound healing and revascularization. However, target cells for the effects of thrombin include not only platelets, endothelial and smooth muscle cells, but also leukocytes, fibroblasts and nerve ending (Figure 7). Thrombin-induced activation of a wide range of cellular responses, including platelet aggregation, leukocyte extravasation, angiogenesis, nerve regeneration, and initiation of immune responses is due to a wide distribution of thrombin receptors in the vasculature (Macfarlane *et al*, 2001; Vergnolle *et al*, 2001).

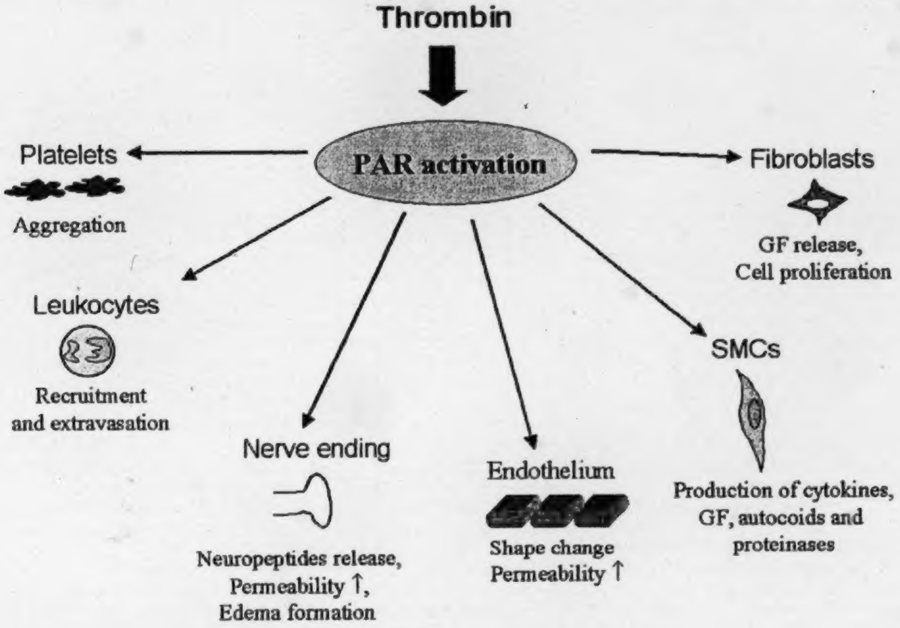

Thrombin
PAR activation
Platelets
Aggregation
Fibroblasts
GF release,
Cell proliferation
Leukocytes
Recruitment
and extravasation
SMCs
Nerve ending
Endothelium
Production of cytokines,
GF, autocoids and
proteinases
Neuropeptides release,
Permeability ↑,
Edema formation
Shape change
Permeability ↑

Figure 7. Actions of thrombin on various cells and tissues (PAR: proteinase-activated receptor; SMCs: smooth muscle cells; GF: growth factor).

Proteinase-Activated Receptors (PARs) and PAR Agonists

A small family of G-protein-coupled receptors called proteinase-activated receptors (PARs) (Figure 8) mediates some of the most important actions of thrombin. Similar to the classical G-protein-coupled receptors, the structure of PARs consists of a seven-transmembrane domain, an intracellular carboxyl terminal and an extracellular amino terminal. The activation of PARs is unique amongst G-protein-coupled receptors in that the process involves a specific proteolytic cleavage at the amino-terminal of the receptors (Macfarlane *et al*, 2001). This proteolysis exposes a new amino-terminal that serves as a tethered ligand, binding intramolecularly to the body of the receptor to stimulate the signal transduction pathway (Vu *et al*, 1991a; Chen *et al*, 1994). Four PARs have been identified in human tissues. PAR1, PAR3 and PAR4 are cleaved and activated by thrombin, while PAR2 is activated by trypsin (Coughlin, 2000; Nakanishi-Matsui *et al*, 2000). Human platelets express PAR1 and PAR4, and the activation of either receptor by PAR agonists triggers platelet secretion and aggregation (Kahn *et al*, 1999).

Specific agonist peptides have been designed for the thrombin-independent activation of PARs. They mimic the tethered ligands, presumably by interacting with receptor extracellular domains and forcing the same conformational changes in the body of the receptor that are needed to activate G-proteins located at the cytoplasmic face of the receptor (Bahou *et al*, 1993; Gerszten *et al*, 1994). Previous studies have shown that these synthetic peptides function as PAR agonists, and generate most of the biological actions of thrombin by a non-proteolytic mechanism (Vu *et al*, 1991a; Vassallo *et al*, 1992). The development of receptor-selective PAR-activating peptides (PAR-APs) makes it possible to investigate the physiological consequences of the activation of particular PARs both *in* *vitro* and *in vivo*. In the present study, the selective PAR1AP (Thr-Phe-Leu-Leu-Arg-amide) and PAR4AP (Ala-Tyr-Pro-Gly-Lys-Phe-amide) were used as the pharmacological probes of PAR functions in human platelets (Chung *et al*, 2002).

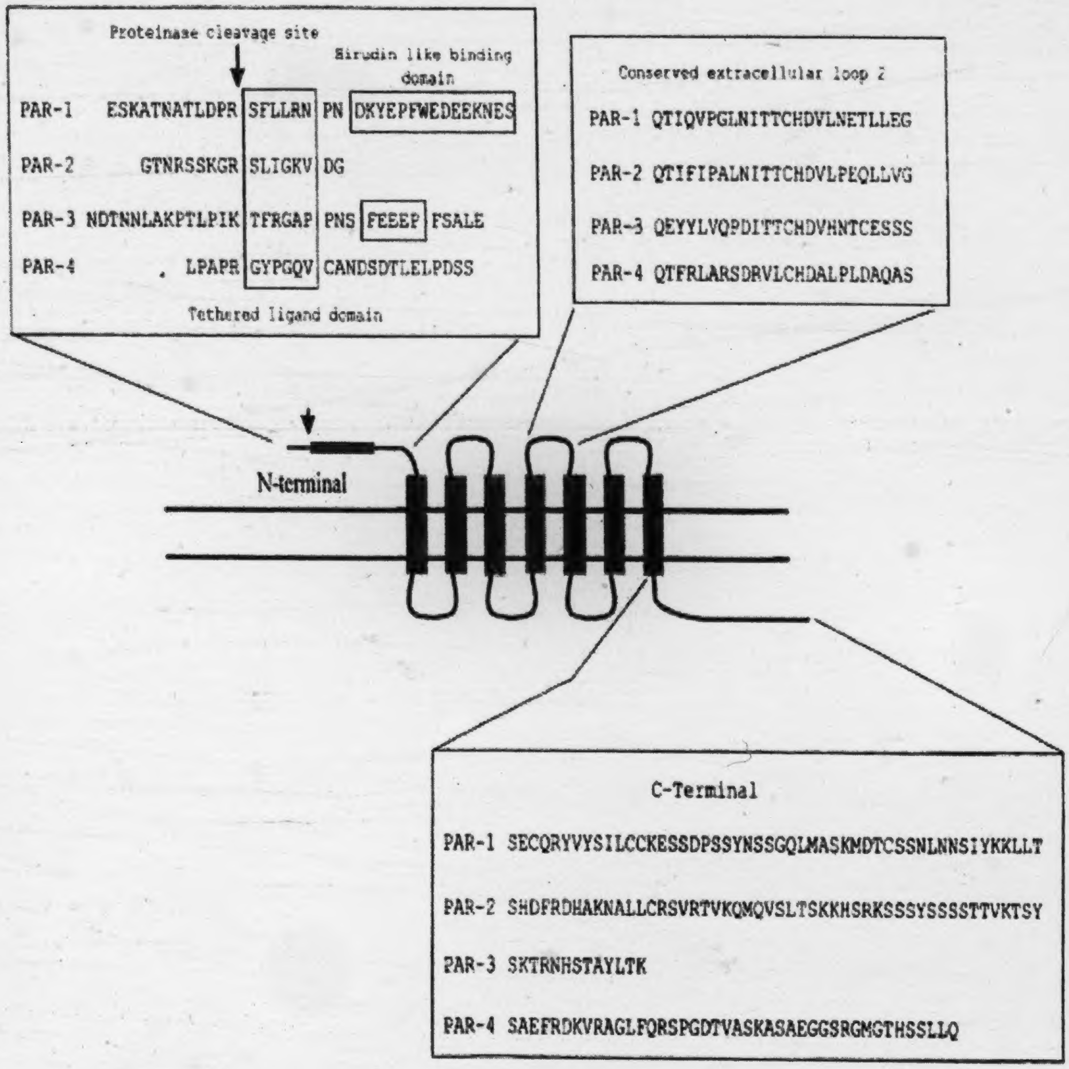

Proteinase cleavage site
Hirudin like binding domain
PAR-1 ESKATNATLDPR SFLLRN PN DKYEPFWEDEEKNES
PAR-2 GTNRSSKGR SLIGKV DG
PAR-3 NDTNNLAKPTLPIK TFRGAP PNS FEEEP FSALE
PAR-4 LPAPR GYPGQV CANDSDTLELPDSS
Tethered ligand domain
Conserved extracellular loop 2
PAR-1 QTIQVPGLNITTCHDVLNETLLEG
PAR-2 QTIFIPALNITTCHDVLPEQLLVG
PAR-3 QEYYLVQPDITTCHDVHNTCESSS
PAR-4 QTFRLARSDRVLCHDALPLDAQAS
N-terminal
C-Terminal
PAR-1 SECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT
PAR-2 SHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY
PAR-3 SKTRNHSTAYLTK
PAR-4 SAEFRDKVRAGLFQRSPGDTVASKASAEGGSRGMGTHSSLLQ

Figure 8. Domain structure of PARs. The key structures for PAR activation are highlightened. The N-terminal consists of proteinase cleavage site and the tethered ligand domain that is unmasked after proteolysis. The N-terminal of PAR1 and PAR3 have the hirudin-like binding domain, which is important for receptor cleavage at low concentrations of thrombin. The conserved extracellular loop 2 in the extracellular domain allows the interaction of tethered ligand and receptor. The C-terminal is involved in receptor phosphorylation, internalization and desensitization.

III. Platelet-Leukocyte Interactions

The interactions between platelets and leukocytes in thrombus formation were observed as early as in 1882 by Giulio Bizzozero who wrote that "The blood platelets which are carried by the blood stream are arrested as soon as they reach the lesion of the arterial wall; at first one observes two to six platelets; very rapidly their number grows into hundreds. Usually, among these, a few white blood corpuscles are also arrested" (Cerletti *et al*, 1992). In physiological conditions, colocalization of platelets and leukocytes at sites of vascular damage is beneficial to wound healing and clotting. However, under pathological situations, platelet-leukocyte aggregation may promote thrombosis and inflammation (Bazzoni *et al*, 1991).

Physiological and Pathological Significance of Platelet-Leukocyte Interactions

Activated platelets have been shown to adhere to many different types of leukocytes: neutrophils, monocytes, eosinophils, basophils and natural killer cells, and an undefined population of lymphocytes (de Bruijne-Admiraal *et al*, 1992). In normal vasculature, platelet-leukocyte aggregation is considered as a part of normal hemostasis with physiological significance. In an injured vessel, platelets recognize vWf in the subendothelium and become activated. The resultant layer of adhering platelets readily interacts with blood leukocytes facilitating white cell emigration into the inflamed area for wound healing, tissue repair, protection from infection and maintenance of vascular integrity (Springer, 1994; Kirton & Nash, 2000) (Figure 9).

Platelet-leukocyte aggregates can also be formed as a part of vascular pathology. Increased neutrophil-platelet adhesion is observed in the circulation of patients with acute myocardial infarction, stroke and post-coronary angioplasty (Michelson, 1996).

Enhanced monocyte and neutrophil adhesion on platelets has also been reported in unstable angina and after cardiopulmonary bypass (Rinder *et al*, 1992; Rogers *et al*, 1998; Ross, 1999). Within atherosclerotic lesions, the stenotic vessels create high shear, which induces platelet activation and interaction with flowing leukocytes (Holme *et al*, 1997), thereby promoting the formation of embolus and the development of infarcts.

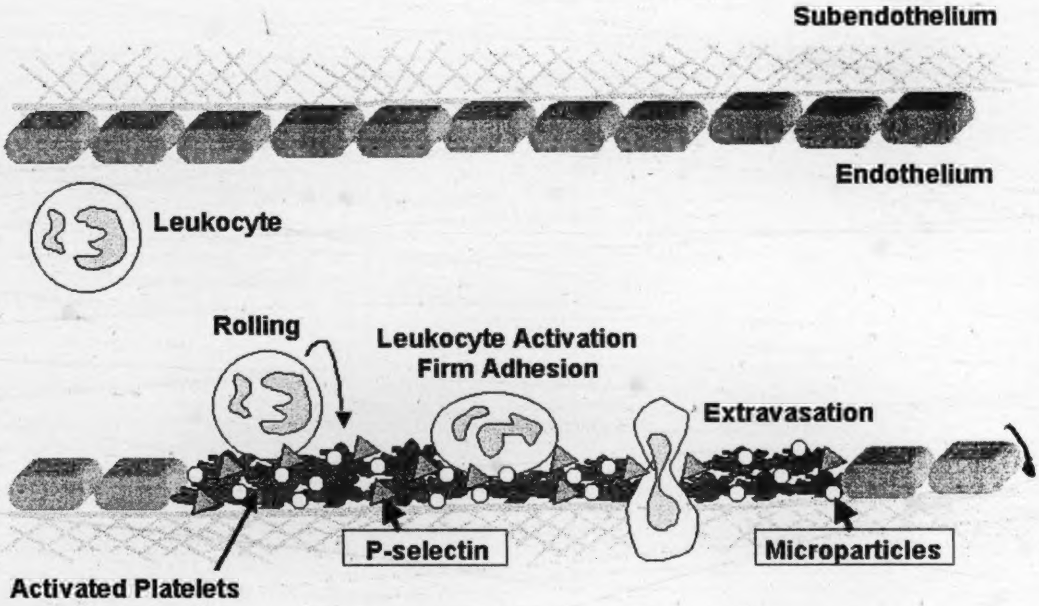

Subendothelium
Endothelium
Leukocyte
Rolling
Leukocyte Activation
Firm Adhesion
Extravasation
P-selectin
Microparticles
Activated Platelets

Figure 9. Sequential events underlying the recruitment of leukocytes from the blood stream to the site of vessel injury. A layer of activated platelets is formed on the subendothelial components. Platelet-derived P-selectin and microparticles support the movement of leukocytes: rolling, adhering and the subsequent transmigration into the damaged vessel.

Platelets and leukocytes modulate the functions of each other (Cerletti *et al*, 1995) (Figure 10). *Leukocytes* stimulate or inhibit platelets, depending on the physiological conditions. Leukocyte-derived NO and ADPase inhibit platelet functions in the same manner as seen for the endothelium-derived NO and ADPase. Elastase, a serine proteinase stored in the azurophilic granules of neutrophils, reduces the binding of thrombin to the platelet surface, and inhibits the GPIb/IX/V-dependent interaction of platelets with vWf (Wicki *et al*, 1985). These effects can be ascribed to a proteolytic downregulation of the activity of GPIb/IX/V complexes. Thus, leukocytes may suppress platelet functions and limit the growth of thrombus. On the other hand, several leukocyte-derived substances activate platelets. Platelet activating factor, a lipid mediator, may amplify platelet activity (Montrucchio *et al*, 2000). A serine protease cathepsin G initiates diverse platelet responses including the elevation of intracellular calcium, secretion, TXA_2 production and downregulation of GPIb/IX/V through proteolytic cleavage and redistribution of the receptor to the intracellular compartments (de Gaetano *et al*, 1999). Interestingly, in the presence of fibrinogen, elastase activates platelets through the enzymatic cleavage of GPIIb/IIIa, inducing a conformational change of the integrin, and leading to its enhanced affinity for fibrinogen binding (Kornecki *et al*, 1986).

Similar to leukocytes, *platelets* contribute to inflammation as they release a numbers of pro-inflammatory substances (Mannaioni *et al*, 1997). They include vasoactive amines (i.e. serotonin and histamine), chemoattractants (i.e. platelet derived growth factor and transforming growth factor β), membrane lipid derivatives (i.e. platelet activating factor and arachidonic acid metabolites) and chemokines (i.e. platelet factor 4 and neutrophil-activating peptide 2). Adhesion of recruited neutrophils can be strengthened by platelet-derived serotonin and TXA_2. Adenosine nucleotides and platelet-derived growth factor are involved in neutrophil degranulation, phagocytosis and respiratory burst (Mannaioni *et al*, 1997). Therefore, activated platelets release substances that attract the circulating leukocytes and accentuate their accumulation at sites of injury. On the other hand, platelet-derived NO and transforming growth factor-β contribute to the platelet-dependent neutrophil inhibition. This helps to counterbalance the harmful effects of excessive neutrophil stimulation, limiting the tissue damage by the uncontrolled production of toxic oxygen-radicals and proteolytic enzymes during prolonged inflammatory conditions.

The platelet-leukocyte interactions not only result in the reciprocal functional modulation, but also in metabolic cooperation (Bazzoni *et al*, 1992; Cerletti *et al*, 1992). A tight contact between platelet and leukocyte membranes creates optimal conditions for exchanging intermediate metabolites and synthesizing substances which can not be made by either cell alone (Fitzpatrick *et al*, 1994). For example, cooperation can take place in the biosynthesis of leukotriene C_4 (LTC_4) (Figure 10). Indeed, arachidonic acid is transferred from platelets to leukocytes, where it is used to synthesize the intermediate LTA_4. LTA_4 is then transferred back to platelets, where it can be metabolized to LTC_4 by platelet glutathione-S-transferase. LTC_4, a potent vaso- and broncho-constrictor, is an important inflammatory mediator (Maugeri *et al*, 1994).

The broad range of interactions between platelet and leukocyte explains how platelet-dependent hemostasis and thrombosis are involved in the inflammatory responses. In order to achieve this cross-talk, adhesive molecules and receptors present on the surface of platelet and leukocyte need to be engaged to stimulate this association.

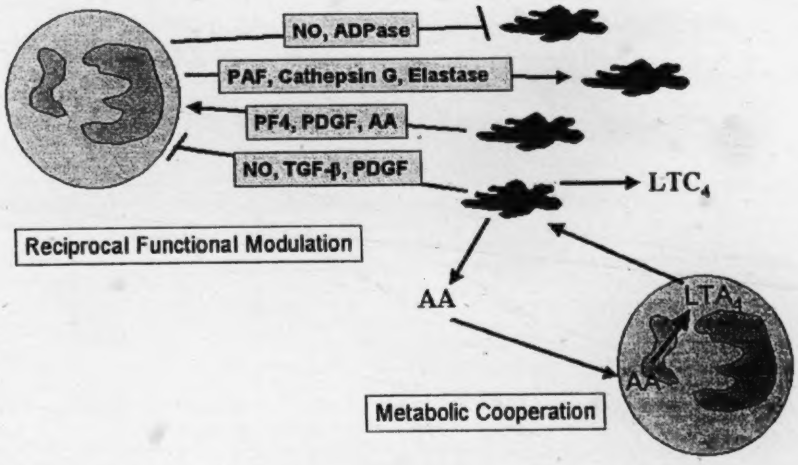

NO, ADPase
PAF, Cathepsin G, Elastase
PF4, PDGF, AA
NO, TGF-β, PDGF
LTC4
Reciprocal Functional Modulation
AA
LTA4
AA
Metabolic Cooperation

Figure 10. Platelet-leukocyte interactions enable the reciprocal functional modulation of these cells through the release of various mediators, and the metabolic cooperation in the biosynthesis of LTC_4.

Molecules and Receptors Involved in Platelet-Leukocyte Interactions: P-selectin and Microparticles

The initial contact between platelets and leukocytes is generally described as 'rolling'. Rolling denotes a type of leukocyte binding in which leukocytes repeatedly tumble end-over-end along the adherent platelet layer (Yang *et al*, 1999). Activated platelets express adhesive molecules of the selectin family that interact with specific carbohydrate ligands on leukocytes surface, tethering the leukocytes briefly to the platelets.

The selectin family consists of three structurally related members with different expression by leukocytes (L-selectin), platelets (P-selectin) and vascular endothelium (E and P-selectin) (Celi *et al*, 1997). They have a characteristic extracellular structure consisting of three domains: a lectin domain, an epidermal growth factor (EGF)-like domain, and a number of repeats related to complement regulatory proteins. P-selectin (CD62-P) is a 140-KDa transmembrane adhesion receptor expressed by platelets and endothelial cells. In resting platelets, it is restricted to the membrane of α-granules. Platelet activation leads to translocation of P-selectin to the plasma membrane following secretion of α-granules. P-selectin consists of an extracellular NH_2-terminal carbohydrate recognition domain, which specifically binds to oligosaccharide-based ligands, such as sialyl-Lewis X in a calcium-dependent manner. Cell adhesion is mediated by the selectin lectin domain and by this specific carbohydrate moiety (Figure 11).

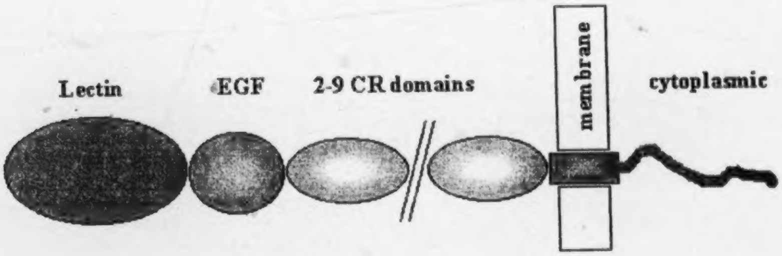


Figure 11. Schematic diagram of P-selectin. P-selectin has an NH_2-terminal carbohydrate-recognition domain like those in C-type lectins, followed by an EGF-like domain, a series of short consensus repeat (CR) domains, a transmembrane domain, and a short cytoplasmic tail.

The major P-selectin counter-receptor on leukocytes is a mucin-like, homodimeric, and sialyl-Lewis X-containing protein called P-selectin glycoprotein ligand-1 (PSGL-1), but L-selectin may also serve a ligand for P-selectin (Sako *et al*, 1993). Since both PSGL-1 and L-selectin are constitutively expressed on leukocytes, P-selectin-mediated attachment of activated platelets does not require prior leukocyte activation. Both P-selectin and PSGL-1 are highly extended glycoproteins, projecting their NH_2-terminal binding sites 40-50 nm above the plasma membrane. This distance is higher than that of most other cell-surface glycoproteins and glycolipids. In addition, as a minor glycoprotein PSGL-1 is concentrated on the tips of microvilli, the region that enhances the ability of adhesion molecules to mediate attachment under flow conditions (Panes & Granger, 1998). Taken together, these physical factors suggest that the orientations of PSGL-1 and P-selectin are ideally suited to optimize their opportunities for interactions under high shear stress (Yang *et al*, 1999).

P-selectin is important in leukocyte recruitment to sites of platelet deposition. P-selectin expressing platelet surface is more adhesive than the endothelium for the localization and recruitment of leukocytes at inflammatory sites, since activated platelets, as compared with endothelial cells, have a higher P-selectin density and a stable functional P-selectin expression (Yeo *et al*, 1994). As mentioned before, a close proximity is required for cell-cell communication via soluble mediators. Membrane interaction creates a microenvironment that maximizes the effects of soluble mediators. For example, neutrophil-derived cathepsin G can be rapidly degraded by antiproteinases in plasma, but P-selectin mediated binding of neutrophils to platelets creates a protected microenvironment that allows cathepsin G to stimulate platelets before being degraded

(Evangelista *et al*, 1991; 1993). Inhibition of platelet-leukocyte interactions with antibodies against P-selectin and PSGL-1 abolishes both reciprocal functional modulation and metabolic cooperation, as shown by decreased synthesis of LTC_4 and TXA_2 (Maugeri *et al*, 1994).

Leukocytes attached to P-selectin on activated platelets encounter juxtacrine activation, which results in their abrupt arrest through the interaction of Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) integrin on leukocytes with the potential ligands on platelets including fibrinogen-bound GPIIb/IIIa, GPIbα, intercellular adhesion molecule (ICAM)-2, factor X, and junctional adhesion molecule-3 (Weber & Springer, 1997; Kuijper *et al*, 1998; Simon *et al*, 2000; Santoso *et al*, 2002). Therefore, PSGL-1 is more than a receptor linking leukocytes to platelets; it can also transduce an 'outside-in' signal to upregulate the adhesive function of Mac-1 integrin (Evangelista *et al*, 1999). P-selectin acts as a first step in slowing leukocytes (Dore *et al*, 1993), so that they become locally activated and adherent via integrin-mediated binding to platelets.

In addition to P-selectin, platelets contribute to platelet-leukocyte interactions by releasing microparticles (MPs) (Ogura *et al*, 2001). Upon platelet activation, plasma membrane is shed into numerous small vesicles (less than 0.1 μm in diameter) (Holme *et al*, 1993). These MPs contain platelet-specific receptors, GPIIb/IIIa, GPIb and P-selectin, thereby support platelet aggregation, platelet/leukocyte-leukocyte interactions, and the interactions with the endothelium. Platelet MPs are highly thrombogenic due to the exposure of negatively charged phosphatidylserine procoagulant surface (Nieuwland *et al*, 1997), suggesting their roles in the activation of the coagulation pathway (Sims *et al*, 1988; Pasquet *et al*, 1996b). In pathology, the formation of MPs is relevant to the clinical states associated with platelet activation, for instance transient ischemic attacks, autoimmune thrombocytopenia, acute coronary syndromes and cardiopulmonary bypass (Michelson, 1996).

Platelet-Leukocyte Interactions in Coronary Artery Bypass Grafting Surgery

The high affinity heterotypic cell-cell interactions such as platelet-leukocyte aggregation not only facilitate the function of platelets and leukocytes in hemostasis and inflammation but also augment vascular occlusion, thereby impairing blood flow and exacerbating ischemia. In the clinical setting, the coronary artery bypass grafting surgery (CABG) is associated with the inflammatory and thrombotic responses, leading to platelet-leukocyte interactions.

In conventional cardiac surgery (i.e. CABG), the patient undergoes cardiopulmonary bypass (CPB) (heart-lung machine), which consists of an oxygenator that exchanges oxygen and carbon dioxide with venous blood, and circuits that supply venous blood to the oxygenator and return arterialized blood to the arterial system of the patient.

The use of CPB is required to arrest the heart, and this is a routine procedure to perform in cardiac surgeries including CABG. Although the advances in CPB have allowed CABG to become one of the most common surgical procedures in Europe and North America, a number of serious complications of CPB have been noted. First, CPB can alter normal hemostatic balance and predispose patients to the risk of excessive postoperative bleeding (Woodman & Harker, 1990). Second, CPB has been associated with decrements in cardiac performance in human and animal studies (Breisblatt *et al*, 1990; Gorcsan *et al*, 1994). Along with cardiac impairment there is an extensive literature documenting a generalized organ injury following CPB including stroke, intellectual deterioration and lung dysfunction (Johnson *et al*, 1996; Roach *et al*, 1996). The pathophysiological rationale for these clinical impairments is not clear. Some of these effects can be attributed to hypothermia and hemodilution, which are integral parts of CPB procedure. Indeed, activation of the coagulation, fibrinolytic, complement, and inflammatory pathways caused by decreased body temperature and blood dilution with plasma expanders or substitutions, may all contribute to postoperative bleeding and other potential complications (Despotis *et al*, 2001) (Figure 12). There is also evidence for activation of the hemostatic system by various humoral pathways (Furie & Furie, 1992). However, there is now general consensus that the lesion of extracorporeal circulation is primarily due to interactions of platelets and white blood cells with the foreign, nonendothelialized surface within the oxygenator and bypass circuit, resulting in factor XII activation and amplification of the intrinsic pathway. In addition to the intrinsic pathway, the extrinsic pathway of coagulation can be also activated by surgery tissue trauma, release of tissue factor, and generation of factor VIIa. The consequence of both pathways is the generation of thrombin, a pivotal enzyme in the control of hemostasis. Thrombin not only converts fibrinogen to fibrin, but also acts on platelets, inflammatory cells, and the endothelium to modulate both hemostasis and inflammation. Thus, it is likely that as a result of initial adhesion signals induced by platelet and leukocyte contacts with the foreign surface pathologic reactions are triggered, and these reactions perpetuate and lead to tissue and organ injury. However, the molecular rationale for platelet dysfunction caused by CPB is still poorly understood and remains to be investigated.

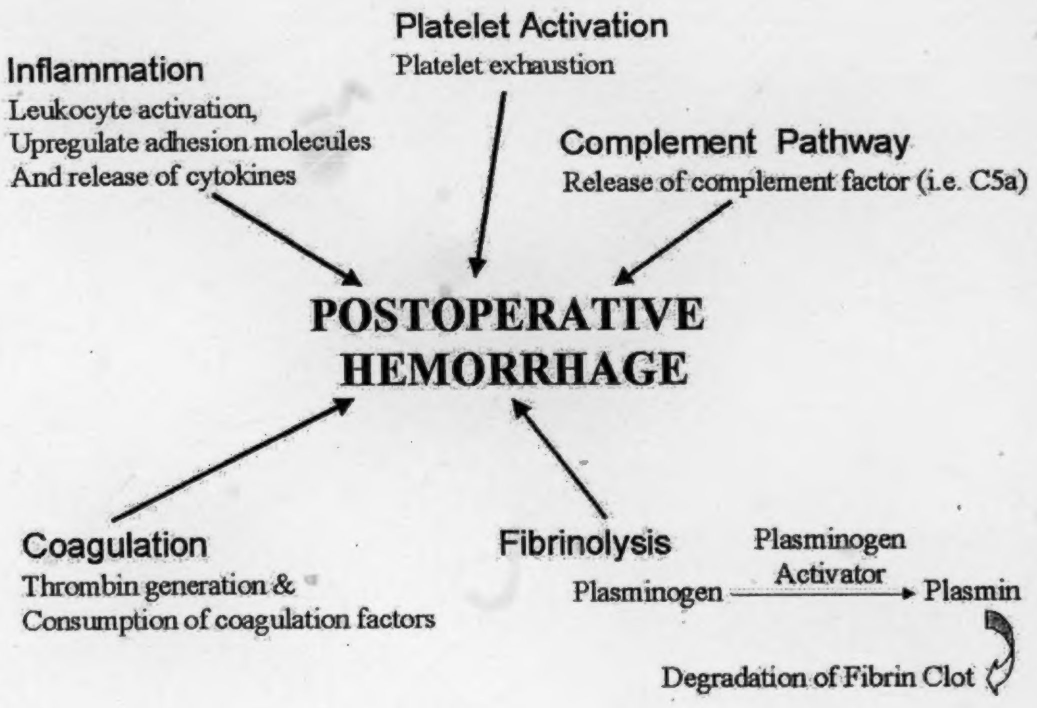

Platelet Activation
Platelet exhaustion
Inflammation
Leukocyte activation,
Upregulate adhesion molecules
And release of cytokines
Complement Pathway
Release of complement factor (i.e. C5a)
POSTOPERATIVE HEMORRHAGE
Coagulation
Thrombin generation &
Consumption of coagulation factors
Fibrinolysis
Plasminogen Activator
Plasminogen
Plasmin
Degradation of Fibrin Clot

Figure 12. Possible mechanisms of CPB-induced postoperative hemorrhage.

Thesis Objective, Hypothesis and Aims

The overall **objective** of my PhD research was to study regulation of functions of platelets and leukocytes by proteinases including proteinase activated receptor (PAR) agonists and matrix metalloproteinases (MMPs) under normal and clinical conditions. It is **hypothesized** that interactions between PAR agonists and MMPs play an important role in platelet and leukocyte functions.

The specific **aims** of my investigations were as follows:

1. To compare the aggregatory potency and the expression of GPIb and GPIIb/IIIa receptors during PAR-mediated platelet activation with different PAR agonists.
2. To investigate platelet-leukocyte interactions by measuring platelet-leukocyte aggregation, P-selectin expression and microparticle generation in the presence of PAR agonists.
3. To study the regulatory effects of prostacyclin and nitric oxide on PAR-mediated platelet aggregation, expression of GPIb and GPIIb/IIIa, and platelet-leukocyte interactions.
4. To investigate contributions of thromboxane A_2-, matrix metalloproteinase-2- and ADP-dependent pathways to PAR-mediated platelet activation and platelet-leukocyte interactions.
5. To determine the significance of leukocyte-derived matrix metalloproteinases in platelet-leukocyte aggregation.
6. To measure platelet-leukocyte aggregation, leukocyte and platelet activation before and after cardiopulmonary bypass (CPB).

7. To investigate the effects of nitric oxide and a stable prostacyclin analogue, iloprost, on CPB-induced platelet activation and platelet-leukocyte interactions in patients undergoing cardiopulmonary artery bypass grafting (CABG).

Materials and Methods

All studies described in the thesis have been approved by the Ethics Committee of the Faculty of Medicine and Dentistry of University of Alberta.

Isolation of Human Platelets and Leukocytes

Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet and leukocyte functions for two weeks prior to the study. Washed platelets (250×10^6 cells/mL) were isolated from blood as described before (Radomski & Moncada, 1983).

Leukocytes were isolated from citrated whole blood using Lympholyte®-poly gradient separating medium (Cedarlane® Laboratories Limited). Blood was layered on the gradient and centrifuged at 500g for 30 minutes at room temperature. The two leukocyte fractions, mononuclear and polymorphonuclear cells, were harvested, washed twice by centrifugation with phosphate-buffered saline (Sigma), and adjusted to 5×10^6 cells/mL using a Coulter counter (Coulter Electronics of Canada Ltd.). Erythrocyte contamination was less than 5% of the total cell number. In this study, the mononuclear and polymorphonuclear cells were pooled and indicated as 'white blood cells' (WBCs) in each experiment.

Subjects and Blood Collection During CABG surgery

33 men and 8 women were included in the study. The clinical characteristics of the recruited patient population are shown in Table 3. Subjects with any disease process affecting platelet function and coagulation disorders, heparin induced thrombocytopenia, chronic inflammatory disorders, chronic renal failure, acute infections, previous open heart surgery or receiving organ transplantation, cognitively impaired, and known

malignancy of less than 5 years were excluded from this study. Informed consent was obtained from each of the participated patients who were randomly assigned to one of the following groups:

A) CPB (control)

B) CPB + Nitric oxide (20 ppm) given into the pump oxygenator

C) CPB + Iloprost (2 ng/kg/min) given into the blood phase of the oxygenator

D) CPB + NO (20 ppm) + Iloprost (2 ng/kg/min)

Table 3. Summary of clinical and surgical data

Characteristics		Control	NO	PGI_2	$NO+PGI_2$
Sample Size		18	9	9	5
Age		61 ± 12	64 ± 8	65 ± 11	63 ± 9
Weight (Kg)		86 ± 12	91 ± 22	90 ± 16	76 ± 21
Height (m)		1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
Ejection Fraction (%)		51 ± 9	43 ± 7	46 ± 15	45 ± 11
Pump Time (mins)		104 ± 16	99 ± 29	80 ± 13	83 ± 20
Clamp Time (mins)		68 ± 18	52 ± 14	49 ± 22	48 ± 12
Flow (L/min)		4.8 ± 0.3	4.6 ± 0.4	4.7 ± 0.4	4.2 ± 0.5
Δ O_2 Pressure	min 1	121 ± 11	123 ± 15	144 ± 47	105 ± 27
	min 30	118 ± 87	128 ± 21	138 ± 20	100 ± 20
	min 60	97.6 ± 33.6	129 ± 22	144 ± 26	104 ± 27
	end	92.1 ± 27.4	128 ± 23	134 ± 20	106 ± 23
Graft no.		3.3 ± 0.6	3.3 ± 0.7	3.4 ± 0.5	3.3 ± 0.5
ICU stay (hrs)		22 ± 3	23 ± 10	23 ± 4	NA

NA: not available. Data are mean ± SEM.

The choice of NO and iloprost dosage used in this investigation were based on the clinical experience of attending anaesthesiologists and surgeons that showed that the administration of NO (20 ppm) or iloprost (2 ng/kg/min) exerted no significant effects on patient hemodynamics.

Drugs were given during CPB. Blood withdrawn from the radial arterial line (9 mL per sample) was collected using 3.15% tri-sodium citrate preparation (9:1 v:v) before and after heparin administration (allowing five minutes for circulation) and after pump (before protamine administration). Whole blood samples were counted by Coulter counter and analyzed within 15 minutes following blood collection using flow cytometry and impedance aggregation.

Platelet Aggregation

Aggregation of washed platelets was measured by light aggregometry (Sawicki *et al*, 1997; Jurasz *et al*, 2001) using a whole blood ionized calcium lumi-aggregometer (Chronolog Corp., Havertown, PA). Washed platelets or platelet-leukocyte suspensions were preincubated at 37°C for 2 minutes and stirred at 900 RPM. Aggregation was initiated by the addition of PAR agonists, and monitored using Aggro-Link software (Chronolog) for at least 9 minutes. For experiments using inhibitors or purified human MMP proteins, aggregation was initiated after 1-2 minutes preincubation (37°C) with these compounds. In the experiments using neutralizing monoclonal antibodies, these reagents were preincubated for 20 minutes at 37°C. (The control experiments, in the absence of antibodies, were also preincubated for 20 minutes at 37°C.) Platelet aggregation was measured as an extent of light transmittance and expressed as a percent change in maximal light transmission, with 100% representing light transmission of platelet medium (Tyrode's solution) alone.

Platelet aggregation in whole blood was measured by the impedance method as described by Cardinal and Flower (1980). Changes in impedance (ohm) across two platinum electrodes immersed in the sample are used as an index of aggregation. Platelet aggregation in whole blood may depend on many factors, for instance, red blood cell count, white blood cell count, fibrinogen and plasminogen content in the plasma (Mackie *et al*, 1984), therefore the results were expressed as a percentage of the maximal responses of patients before and after treatments where each patient served as his/her own control.

Measurement of ADP Release by Chemiluminescence

ADP secreted from dense granules of stimulated platelets was measured by a whole blood ionized calcium lumi-aggregometer as previously described (Radomski *et al*, 1992; Sawicki *et al*, 1997). Platelets were first preincubated with luciferin-luciferase reagent (440 luciferase units/mL and 4 μg/mL of luciferin) for 2 minutes at 37°C in order to facilitate the conversion of ADP to ATP and to generate chemiluminescence. Afterwards, the agonist was added and the changes in luminescence were monitored. To quantify the generation of platelet ATP, calibration curves were constructed with standard ATP. The data were expressed as nM ATP.

Measurement of Oxygen-Derived Reactive Species during Platelet-Leukocyte Interactions

The generation of oxygen-derived reactive species, an indicator of respiratory burst, was measured simultaneously with platelet aggregation with the addition of 50 μM luminol (Sigma) to the sample. The respiratory burst was expressed as the percent of luminescence which is an arbitrary value.

Flow Cytometry

Platelet activation can be quantified by flow cytometry using specific fluorescence-conjugated antibodies against major platelet receptors. Figure 13 shows schematic representation of platelet activation involving GPIb, GPIIb/IIIa and P-selectin.

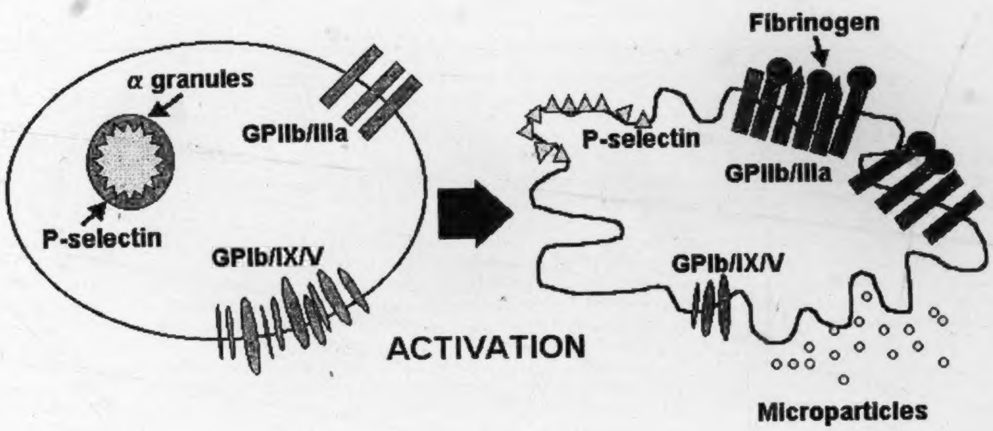

α granules
GPIIb/IIIa
P-selectin
GPIb/IX/V
ACTIVATION
Fibrinogen
P-selectin
GPIIb/IIIa
GPIb/IX/V
Microparticles

Figure 13. Schematic representation of platelet activation. Major platelet activation markers are: (1) internalization of GPIb/IX/V complex, (2) translocation of P-selectin to surface plasma membrane from α-granules, (3) Shedding of plasma membrane into microparticles and (4) upregulation of GPIIb/IIIa complex by increasing copies of receptors on the surface and exposing binding sites for ligands, such as fibrinogen.

In order to analyze the expression of GPIb, total GPIIb/IIIa (both non-activated and activated receptors) and P-selectin on the surface of individual platelets, and to minimize platelet aggregation induced by preparative procedures, no stirring or vortexing steps were used. Platelets were first activated with agonists for 10 minutes, then diluted ten times with physiological saline. In some experiments, platelets were preincubated with inhibitors for 1-2 minutes prior to the addition of agonists. Samples were then incubated in the dark without stirring for 15 minutes at room temperature in the presence of saturating concentrations of phycoerythrin (PE)-labelled GPIb-specific monoclonal antibody (MoAb) (CD42-PE; DAKO Diagnostics Canada Inc.), P-selectin-specific MoAb (CD62P-PE; Becton Dickinson), fluorescein-isothiocyanate (FITC)-labelled GPIIb-specific MoAb (CD41-FITC; DAKO Diagnostics Canada Inc.), or MoAb directed against activated GPIIb/IIIa (PAC-1-FITC; Becton Dickinson). Following incubation, platelets were diluted in FACS Flow fluid and analyzed within 15 minutes by flow cytometer (Becton Dickinson, Mountainview, CA) (Figure 14).

The instrument was set up to measure the size (forward light scatter), granularity (side light scatter) and platelet fluorescence. In the flow cytometry analysis, a two-dimensional analysis gate of forward and side scatter was drawn to include single platelets in an unstimulated sample and exclude platelet aggregates and microparticles. The fluorescence was analyzed using a logarithmic scale, and a fluorescence histogram was obtained for 10,000 individual events (Figure 15). GPIb, GPIIb and P-selectin dependent fluorescence was expressed as the mean fluorescence intensity (MFI) of the cell population, as analyzed using CELLQUEST software and expressed as arbitrary units of fluorescence or normalized to control.

Microparticle population was gated by a forward scatter cutoff that was set to the immediate left of the intact platelet population in an unstimulated sample (Figure 16). Microparticles were reported as the percent of PE-positive cells in the gated region of the total counted events.

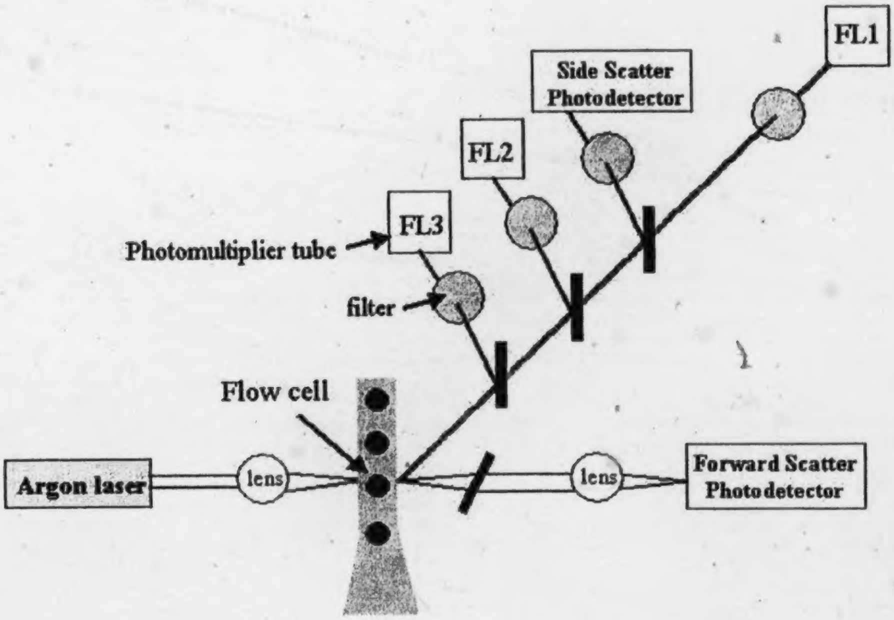

FL1
Side Scatter Photodetector
FL2
FL3
Photomultiplier tube
filter
Flow cell
Argon laser
lens
lens
Forward Scatter Photodetector

Figure 14. Basic principles of flow cytometry. Flow cytometry involves the study of cells under laminar flow conditions. The cytometer is equipped with a 488 nm wavelength argon laser, and 525 nm and 575 nm band pass filters are used for detecting FITC- and PE-fluorescence (FL1 and FL2).

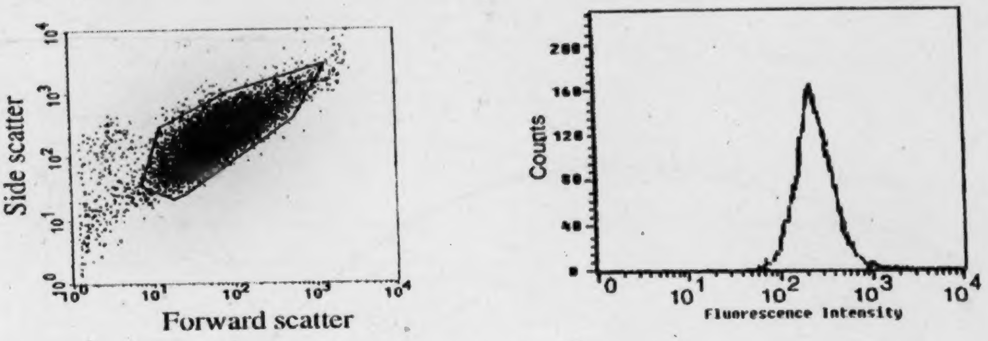


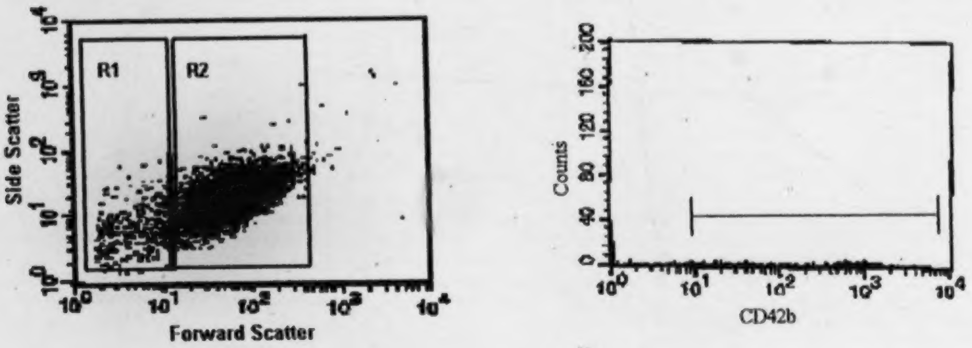
Figure 15. Flow cytometric analysis of platelet surface expression of GPIIb. Left panel is the scatter plot showing the light scattering properties of 10000 counted events. A 2-dimentional gate is set to include individual platelets in an unstimulated sample and exclude microparticles and platelet aggregate. A histogram plot (right panel) is drawn to analyze the fluorescence intensity, which represents the receptor expression level. (Expression of GPIb and P-selectin is analyzed by the same way).

Platelet-leukocyte aggregates (PLA) were detected by dual colour flow cytometry. After aggregation experiments, samples were incubated with FITC-conjugated MoAbs directed against human leukocyte common antigen (CD45) (CD45-FITC; Becton Dickinson) and CD62P-PE to label leukocytes and platelets, respectively. However, using P-selectin as a platelet marker to measure PLA could potentially underestimate PLA numbers since P-selectin is involved in the binding of platelets to leukocytes. Therefore, some P-selectin molecules might be inaccessible to the identifying antibodies. In an attempt to address this, GPIb and GPIIb/IIIa were also used as platelet markers in preliminary experiments. The estimate of PLA formation was similar using any of the three platelet markers; therefore, P-selectin was used in all subsequent experiments. After incubation, samples were analyzed by gating leukocytes, based on their FITC-CD45 fluorescence and light scatter profile, and quantifying the platelet fluorescent signal associated with leukocytes. Formation of PLA was expressed as the percent of leukocytes exhibiting platelet-CD62P fluorescence relative to the total counted events (Figure 17).

Membrane surface expression of MMP-1, MMP-2, MMP-3 and MMP-9 was analyzed by flow cytometry as described by Kazes *et al* (2000), with the use of human MoAbs to MMP-1 [Ab-6], MMP-2 [Ab-6], MMP-3 [Ab-5] and MMP-9 [Ab-1] (Oncogene) as the primary antibodies and FITC-conjugated goat anti-mouse IgG specific polyclonal antibody (Becton Dickinson) as the secondary antibodies.

Whole blood flow cytometry was performed with blood samples collected from patients at the onset (one sample was collected before the administration of heparin, and one was collected afterward) and at the termination (pump off, before protamine infusion) of CPB. Platelet receptors, GPIb, total GPIIb/IIIa and P-selectin, microparticles and PLA were analyzed by flow cytometry as described above. The PLA count was expressed as 10^9/L, which was calculated by multiplying the percent of PLA in the total counted CD45-positive events by the number of leukocytes in the whole blood measured with Coulter counter. The expression of leukocyte Mac-1 (CD11b integrin) was measured by dual leukocyte labelling with CD45-FITC and CD11b-PE.

Unstimulated Samples



1 nM Thrombin

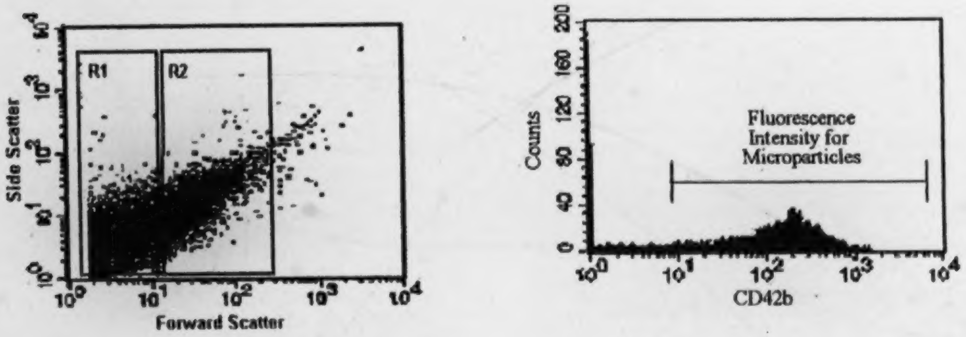


Figure 16. Flow cytometric analysis of platelet-derived microparticles. Scatter plots (left panels) showing the light scattering properties of 10000 counted events of an unstimulated sample and a thrombin (1 nM)-stimulated sample. Since the generation of microparticle is an activation-dependent process, the microparticle population is distinguished and gated (as shown as gate R1) based on the forward scatter cutoff that is set to the immediate left of the individual platelet population (as shown as gate R2) of unstimulated sample. With the use of PE-labelled anti-CD42b antibodies, the corresponding histogram plots (right panels) show the fluorescence intensity of gate R1. Microparticles are reported as a percent of PE-positive events in gate R1.

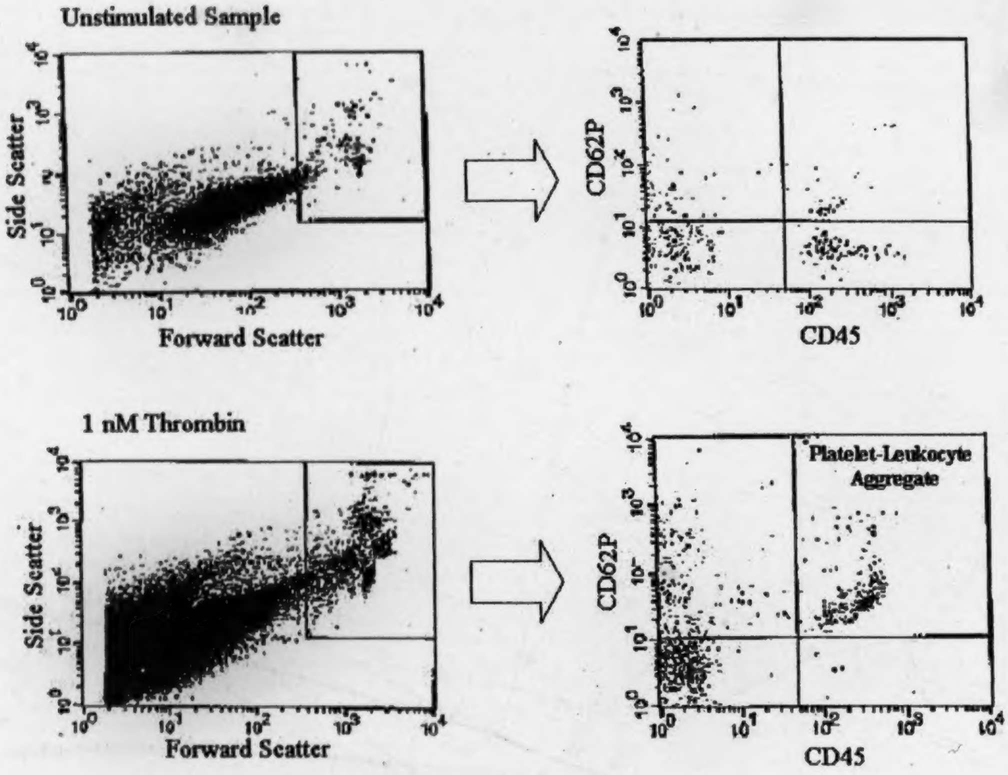


Figure 17. Flow cytometric analysis of PLA. Scatter plots (left panels) showing the light scattering properties of 10000 events of an unstimulated sample and thrombin (1 nM)-stimulated sample. The population of PLA is distinguished and gated by the forward scatter cutoff that is set to the immediate left of the individual platelet population of an unstimulated sample. With the use of PE-labelled anti-CD62P and FITC-labelled anti-CD45 antibodies, the corresponding 2-parameter scatter plots (right panels) show the percent of PLA, positive in both PE- and FITC-fluorescence, in the 10000 counted events.

Western Blotting

Following aggregation, samples of platelet-leukocyte suspensions were collected and centrifuged at 1000g for 10 minutes at room temperature. The resultant supernatant and pellet portions were studied for the presence of MMP-1, -2, -3 and –9 using Western blot analysis. Western blot analysis of MMP-2 and MMP-9 were performed as previously described by Fernandez-Patron *et al* (1999a), except for the use of MoAbs against MMP-2 and MMP-9 that were obtained from Oncogene (Boston, MA). For the analysis of MMP-1 and MMP-3, samples were subjected to 12% SDS-PAGE, and the blots were probed with MoAbs against MMP-1 and MMP-3 (Oncogene). These MoAbs recognize both the latent and active forms of MMPs. The immunoreactive bands were detected with an ECL kit (Amersham), read and quantified by densitometry using a ScanJet 3c scanner (Hewlett Packard) and SigmaGel software (Jandel). Data were expressed as arbitrary units per μg of loaded protein.

Measurement of MMP-2 and MMP-9 Activity

The gelatinolytic activities of MMP-2 and MMP-9 during platelet-leukocyte aggregation were measured by zymography as previously described (Sawicki *et al*, 1997). Briefly, both releasate and platelet-leukocyte homogenate were collected after platelet-leukocyte aggregation. Zymography was performed by subjecting samples to 8% SDS-PAGE with copolymerized gelatin (2 mg/mL; Sigma) as substrate. After electrophoresis, the gels were washed with 2% Triton X-100, and then incubated in incubation buffer (50 mM Tris-HCl buffer with 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3, pH 7.5) at 37°C until the activities of the enzymes could be determined. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 (Sigma) in a mixture of methanol: acetic acid: water (2.5:1:6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue-stained gelatin. Enzyme activities were quantified by using a ScanJet 3c scanner (Hewlett Packard) and SigmaGel measurement software (Jandel), and expressed as arbitrary units per μg of loaded protein.

Measurement of MMP-1 and MMP-3 Activity

To quantify the enzymatic activities of type I collagenase (MMP-1) and stromelysin (MMP-3) in platelets and leukocytes, activity, assay kits were purchased from Chemicon and the measurements performed according to the instructions provided by the manufacturer. Briefly, following aggregation, samples of platelet-leukocyte suspensions were collected and centrifuged at 1000g for 10 minutes at room temperature. The cellular pellets were immediately homogenized by sonication (Vibra Cell) in homogenizing buffer (50 mM Tris-HCl buffer with 3.1 mM sucrose, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL SBTI, 2 μg/mL aprotinin and 0.1% Triton X-100, pH 7.4) and centrifuged (10000g for 10 minutes at 4°C), and the soluble fractions were stored at -80°C until assayed for the presence of MMP-1 and MMP-3. Both cell-associated and secreted metalloproteinases were subsequently measured by enzyme activity kits. The activity of MMP-1 and MMP-3 was expressed as ng per μg of total protein in the sample or normalized to the control level, respectively.

Measurement of cAMP and cGMP

The levels of cAMP and cGMP in blood cells and plasma samples from the clinical study were determined using cAMP/cGMP enzyme immunoassays according to manufacturer's instructions (Assay Designs). Briefly, within 10 minutes of blood collection, 50 mM

EDTA and 50 mM 3-Isobutyl-1-methylxanthine (IBMX, Sigma) were added to the whole blood samples to inhibit the activities of phosphodiesterases. The samples were centrifuged (1000g) at 4°C for 10 minutes, and cell-free plasma was frozen at -80°C and stored until assayed. Blood cells were lysed by sonication (Vibra Cell); samples were centrifuged and supernatants were used to measure the cyclic nucleotides. The variation of the levels of cAMP and cGMP in samples from each subject was large; therefore, results were expressed as a percentage of the values from the pre-heparin samples of the corresponding subjects.

Microscopy

The platelet-leukocyte samples were collected from the aggregometer cuvette at the end of aggregation. The platelet-leukocyte interactions were viewed using a Leitz phase-contrast microscope equipped with a Sony colour video camera connected to a Samsung video recorder.

Reagents and Peptides

Collagen, thrombin, ATP standard and luciferin-luciferase reagent were obtained from Chronolog (Havertown, PA). Apyrase, aspirin, prostacyclin, S-nitrosoglutathione, phenanthroline, and amastatin, were purchased from Sigma Chemical Co (St. Louis, MO). 1H-[1,2,4]Oxadiazole[4,3]quinoxalin-1-one (ODQ) was from Tocris Cookson Ltd. TRAP (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-amide) was purchased from Sigma. PAR1AP (Thr-Phe-Leu-Leu-Arg-amide) and PAR4AP (Ala-Tyr-Pro-Gly-Lys-Phe-amide) were synthesized by the Alberta Peptide Institute (University of Alberta, Canada). The purity (> 95% by HPLC) and composition of PAR1AP and PAR4AP were verified by mass spectrometry and the concentrations of stock solutions dissolved in 25 mM HEPES buffer were measured by quantitative amino acid analysis. All other reagents were analytical grade.

Statistical Analysis

All results were given as mean ± s.e.mean (SEM) of at least three independent experiments. Data were analyzed by paired or unpaired Student's t-test, one-way analysis of variance (ANOVA) with Dunett's post test for selective comparisons, repeated measures ANOVA and two-way ANOVA, where appropriate, using Prism software (GraphPad, San Diego, CA). The EC_{50} and IC_{50} values were also calculated using Prism software. P values of less than 0.05 were considered to be statistically significant.

Results

Mechanisms of Action of Proteinase-Activated Receptor Agonists on Human Platelets

Effects of PAR agonists on platelet aggregation

Figure 18A shows concentration-response curves for thrombin, TRAP, PAR1AP and PAR4 AP. The order of aggregatory potency was: thrombin > PAR1AP >TRAP > PAR4AP, as determined by EC_{50} values of 0.29 nM ± 0.00, 3.9 μM ± 1.1, 24 μM ± 2 and 60 μM ± 2, respectively, $p<0.05$, $n = 5$. Subthreshold concentrations of PAR1AP (3 μM) potentiated the effects of PAR4AP (30 μM) to promote maximal and irreversible platelet aggregation (Figure 18B).

The addition of aminopeptidase inhibitor, amastatin (10 μM), did not significantly affect ($p>0.05$; $n = 4$) the aggregatory activity of PAR agonists (EC_{50}s for PAR agonists in the presence of amastatin were: TRAP = 24 μM ± 2, PAR1AP = 3.7 μM ± 1.6, PAR4AP = 58 μM ± 3).

Expression of glycoprotein receptors during PAR-agonist stimulation

Stimulation of platelets with thrombin, TRAP, PAR1AP, PAR4AP, as well as with the potentiating combination of PAR1AP and PAR4AP resulted in a significant down-regulation of expression of GPIb (Figure 19A). This reduction was approximately 40% of GPIb levels in unstimulated platelets and was similar for all tested agonists.

In contrast, thrombin and PARAP-induced platelet activation were associated with up-regulation of GPIIb/IIIa expression. Using antibody directed against GPIIb that does not differentiate between the activated and non-activated conformation of GPIIb/IIIa receptors, it was found that PAR agonists resulted in approximately a 50% increase in the

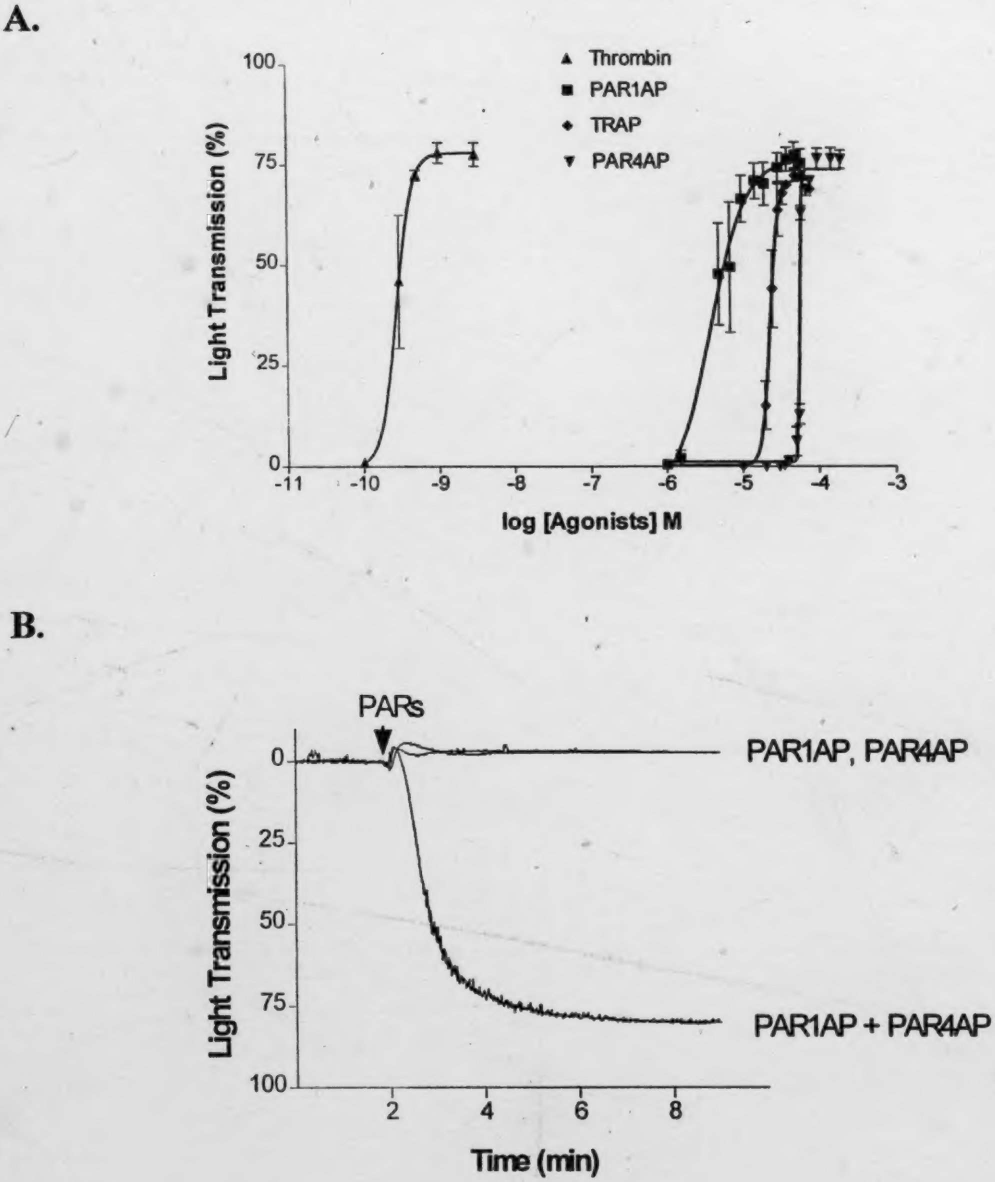


Figure 18. PAR agonist-induced platelet aggregation. Panel A shows concentration-response curves for PAR agonists. Data are mean ± SEM, n = 5. Superimposed aggregatory tracings (panel B) demonstrate amplification of platelet aggregation by the combination of subthreshold concentrations of PAR1AP and PAR4AP.

surface expression of this receptor (Figure 19B). This up-regulation was similar for all agonist treatments. However, the expression of activated GPIIb/IIIa, as measured with selective (differentiating) PAC-1 antibody, was increased 20-80 times compared to control (Figure 19B).

Effects of PGI_2 and GSNO on PAR agonist-stimulated platelet aggregation and GPIIb/IIIa upregulation

Incubation of platelets with PGI_2 or GSNO resulted in a concentration-dependent inhibition of platelet aggregation induced by PAR agonists. Prostacyclin was more potent than GSNO as an inhibitor of PAR agonist-induced aggregation (Table 4 and Figure 20). Platelet aggregation induced by synthetic PAR agonists was more sensitive to inhibition with PGI_2 than those induced by thrombin and collagen (Table 4). These differences were not detected for inhibition of PAR agonist-induced aggregation by GSNO.

Prostacyclin and GSNO inhibited increases in activated and non-activated GPIIb/IIIa in some, but not all PAR agonists (Figure 21). This inhibition was more pronounced for the activated GPIIb/IIIa ($p<0.05$, $n = 3$-9).

The effects of aspirin, phenanthroline and apyrase on PAR agonist-induced aggregation

To study the dependence of PAR agonist-induced aggregation on the activation of the TXA_2, MMP-2 and ADP-mediated pathways of aggregation, respective inhibitors of these pathways such as aspirin, phenanthroline and apyrase (Sawicki *et al*, 1997; Jurasz *et al*, 2001; Radomski *et al*, 2001) were used. ASA exerted no significant effects on thrombin or PAR-AP-induced aggregation (Figure 22). Phenanthroline partially inhibited thrombin-, PAR1AP-, PAR1AP + PAR4AP- and TRAP-, but not PAR4AP-induced

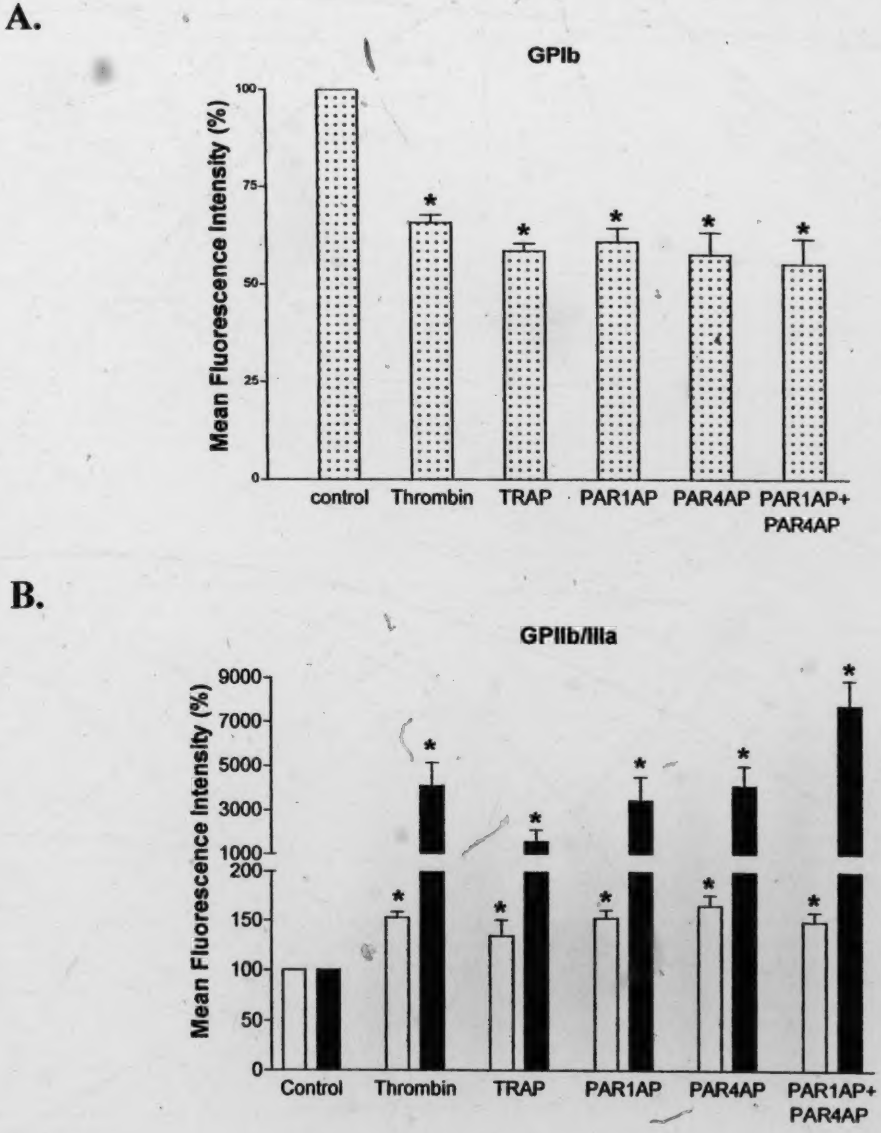


Figure 19. Flow cytometry analysis of GPIb, GPIIb/IIIa and activated GPIIb/IIIa on platelets stimulated with PAR agonists. PAR agonists result in a significant reduction of GPIb (panel A) and up-regulation of total GPIIb/IIIa (open bars) and activated GPIIb/IIIa (closed bars) (panel B). PAR agonists were used at concentrations that resulted in submaximal (95%) aggregatory response (Table 4). Control: unstimulated platelets. Bars are mean ± SEM, n = 3-9. * $p<0.05$ treatments versus control (ANOVA plus post-tests).

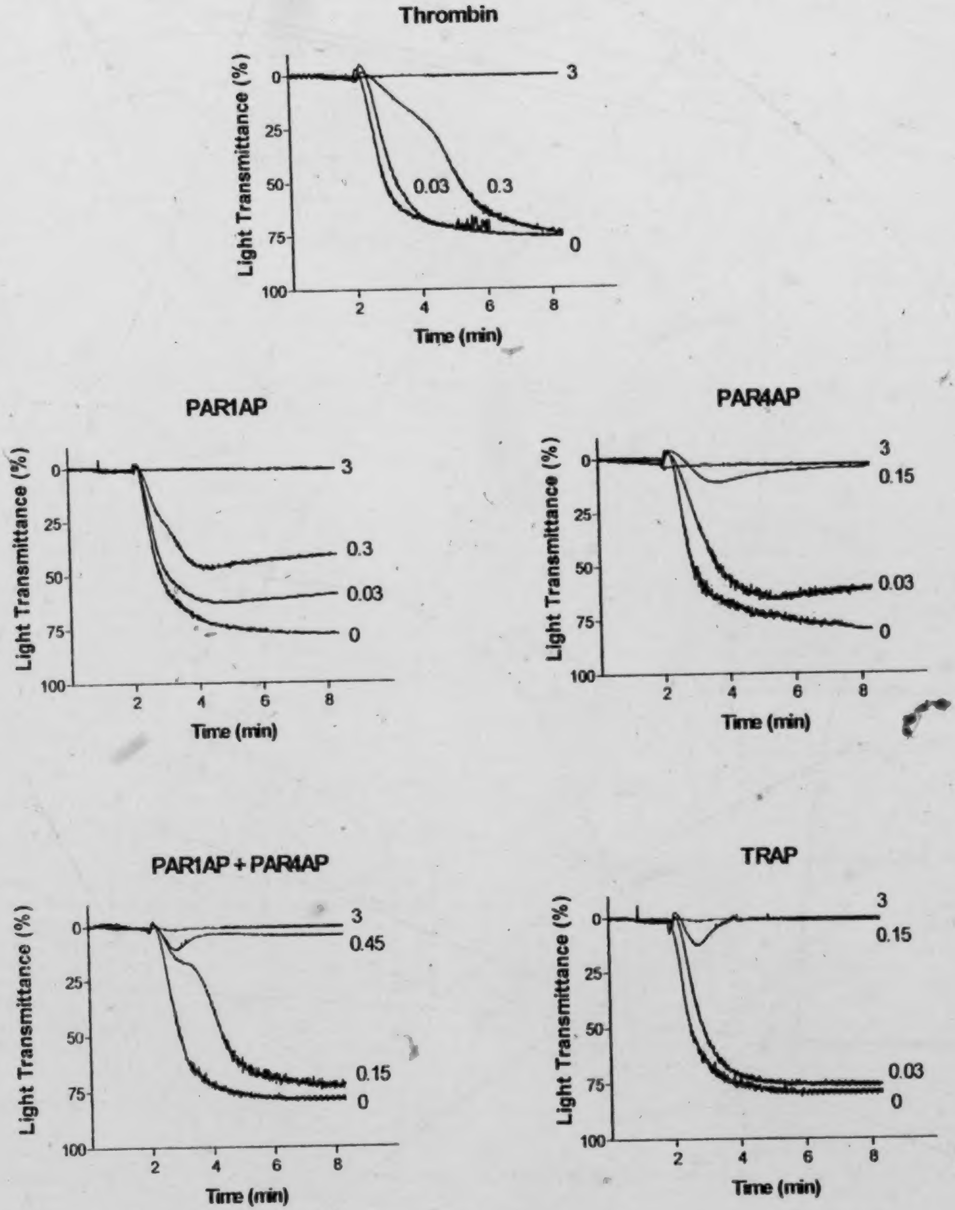


Figure 20. Inhibition of PAR agonist-induced aggregation by PGI_2. Aggregation was induced by submaximal concentrations of PAR agonists. Numbers beside the tracings show the concentrations of PGI_2 in nM. Traces are representative of 3-7 independent experiments.

Table 4. IC_{50} for inhibition of PAR agonist- and collagen-induced aggregation by PGI_2 and GSNO

Aggregatory agonists	PGI_2 (nM)	GSNO (μM)
Thrombin (1 nM)	0.93 ± 0.02	0.34 ± 0.16
TRAP (35 μM)	0.08 ± 0.01 *	0.11 ± 0.11
PAR1AP (10 μM)	0.19 ± 0.14 *	0.21 ± 0.15
PAR4AP (70 μM)	0.09 ± 0.15 *	0.18 ± 0.08
PAR1AP (3 μM)+ PAR4AP (30 μM)	0.12 ± 0.10 *	0.11 ± 0.09
Collagen (4 μg/mL)	0.73 ± 0.02	0.33 ± 0.09

Data are IC_{50} representing mean ± SEM, n = 3-7. * $p<0.05$ PARAPs v/s thrombin or collagen.

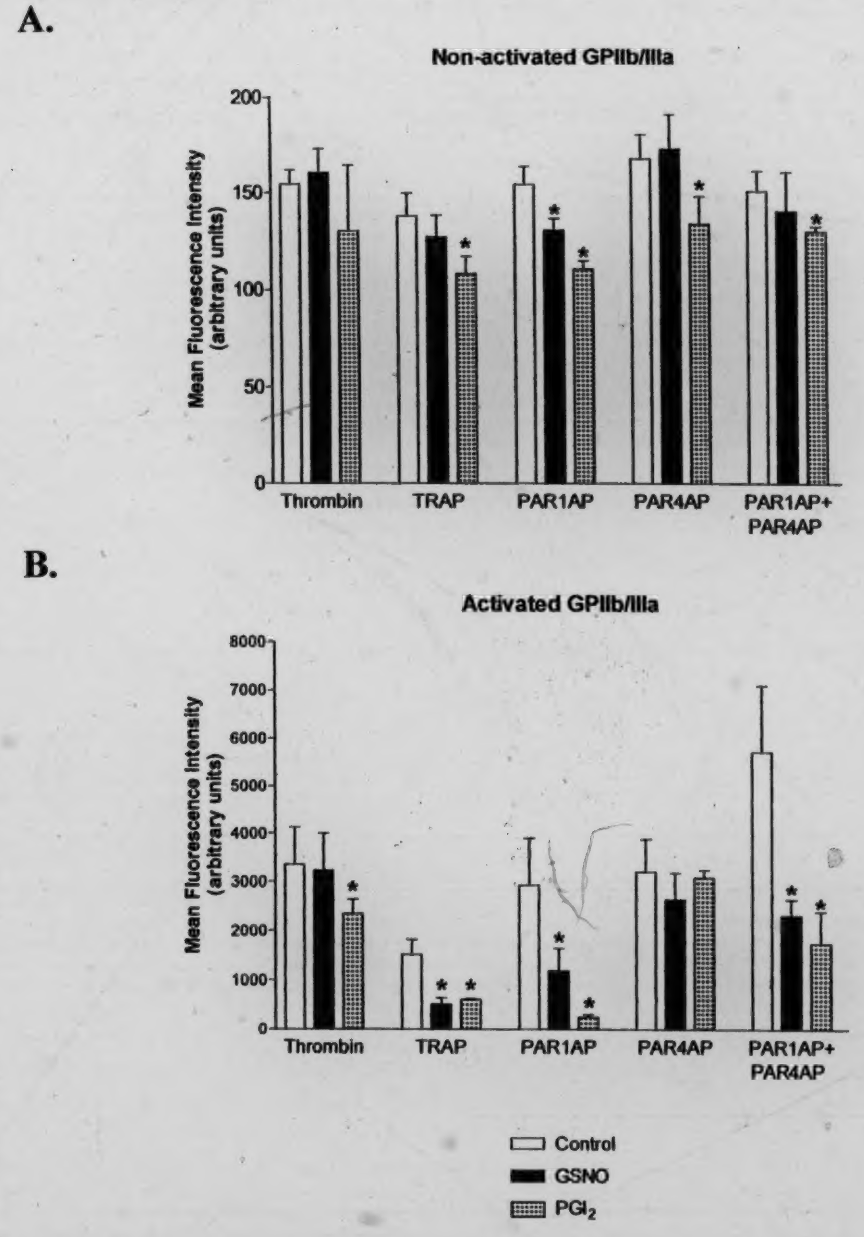


Figure 21. Flow cytometry analysis of non-activated GPIIb/IIIa (panel A) and activated GPIIb/IIIa (panel B) in platelets stimulated with submaximal concentrations of PAR agonists in the presence of 5 μM GSNO (closed bar) or 3 nM PGI_2 (dotted bar). Data are mean ± SEM, n = 3-9. * $p<0.05$ treatments versus control (PAR agonists in the absence of inhibitors, open bar).

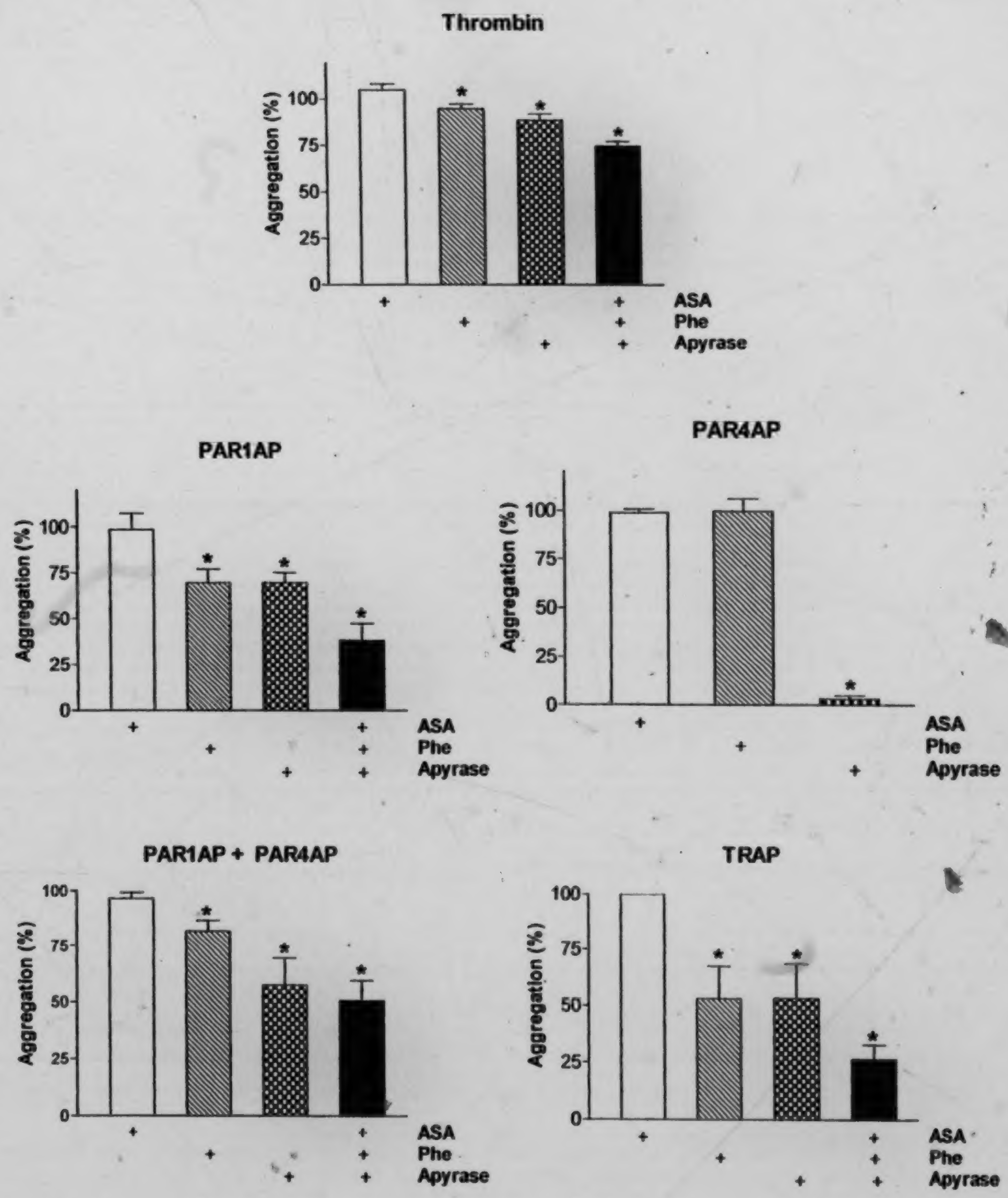


Figure 22. Effects of aspirin (ASA, 300 μM), phenanthroline (Phe, 100 μM) and apyrase (300 μg/mL) on PAR agonist-induced aggregation. Results are expressed as a percent of aggregation induced by the corresponding agonist without inhibitors. Data are mean ± SEM, n = 3-9. * $p<0.05$ treatments versus controls with PAR agonists.

aggregation. The effects of apyrase were most pronounced against PAR4AP-induced aggregation. Moreover, PAR4AP-induced aggregation was abolished by the inhibitor. A combined administration of these inhibitors enhanced the effects of single compounds (Figure 22).

Roles of ADP in PAR4-mediated platelet aggregation

Since PAR4AP-induced aggregation was entirely apyrase-sensitive, we further studied the contribution of ADP pathway to this reaction. PAR4AP-induced aggregation was associated with the generation of ATP and there was a significant correlation ($r^2 = 0.95$, $p<0.05$, $n = 3$) between inhibition of aggregation and reduction of ATP release caused by apyrase during PAR4AP-mediated aggregation (Figure 23A-C). Figure 23D shows that apyrase abolished PAR4AP-mediated activation of GPIIb/IIIa. Ticlopidine, an ADP receptor antagonist, suppressed PAR-4-mediated aggregation, but the inhibitory effect was reversed by the preincubation of a non-aggregatory concentration of epinephrine (Figure 23E).

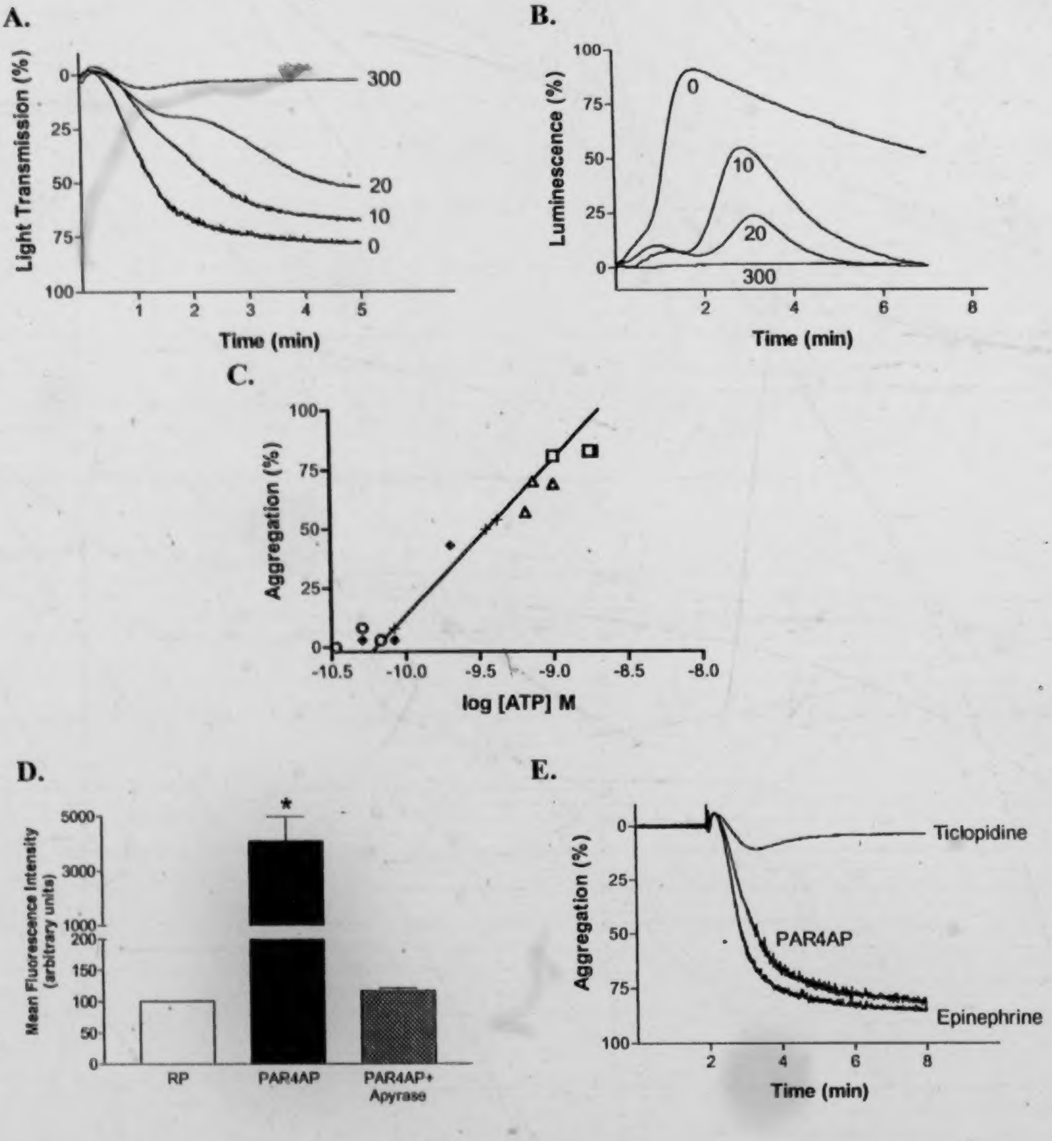


Figure 23. Role of ADP in PAR4-mediated platelet responses. Superimposed aggregatory and chemiluminescence tracings showing apyrase inhibition (0-300 μg/mL) on PAR4AP-induced aggregation (A) and ADP release (B). Correlation between ATP concentrations and aggregation with apyrase inhibition (C; square: 0, triangle: 10, cross: 20, diamond: 35, circle: 300 μg/mL). Apyrase (50 μg/mL) inhibition on GPIIb/IIIa upregulation (D). Epinephrine (10 μM) reverses ticlopidine (80 μM) inhibition on platelet aggregation (E, tracings are representative of 3 similar experiments). Data are mean ± SEM, n = 5. * $p<0.05$ treatments versus RP.

Platelet-Leukocyte Interactions Induced by PAR Agonists

Leukocytes modify PAR agonist-induced platelet aggregation

Figure 24A illustrates the potentiating effect of leukocytes on platelet aggregation induced by PAR agonists at a physiological ratio (platelets: leukocyte ratio = 60:1). The presence of leukocytes shifted the concentration-response curve of thrombin to the left without changing the maximal aggregation response, indicating that leukocytes potentiate thrombin-induced aggregation. The EC_{50}s and EC_{95}s values for PAR agonist-induced aggregation, at the 60:1 platelet:leukocyte ratio, are presented in Table 5.

Figure 24B shows the effect of various concentrations of leukocytes on platelet aggregation induced by thrombin. Leukocytes exerted a biphasic effect on aggregation; at high platelet: leukocyte ratio (100:1), leukocytes potentiated platelet aggregation. This effect was accompanied by increased respiratory burst as measured by luminol-enhanced chemiluminescence. In contrast, at low ratio (5:1) leukocytes abolished aggregation and decreased the respiratory burst. The inhibitory effects of leukocytes on platelet aggregation were prevented by the addition of ODQ, an inhibitor of soluble guanylyl cyclase (Garthwaite *et al*, 1995) (Figure 25). Similar results were obtained with PAR1AP and PAR4AP (data not shown).

Optical aggregometry measures the extent of platelet-leukocyte aggregation; however, this method does not differentiate between platelet-leukocyte and platelet-platelet aggregates. Therefore, I used flow cytometry in order to study, in a selective manner, the formation of platelet-leukocyte aggregates (PLA) and the involvement of cell receptors in this process. Platelets and leukocytes were double-labelled with the respective fluorescence-conjugated antibodies and PLA was analyzed by flow cytometry. Thrombin resulted in a concentration-dependent PLA formation (Figure 26). Similar results were obtained with the addition of other PAR agonists over a range of concentrations (PAR1AP = 0.5-10 μM; TRAP = 1-35 μM; PAR4AP = 10-70 μM).

Table 5. EC_{50} and EC_{95} values of PAR agonist-induced platelet aggregation in the presence of leukocytes. The platelet:leukocyte ratio in these experiments was 60:1.

	EC_{50}		EC_{95}	
PAR Agonist	Control	WBCs	Control	WBCs
Thrombin (nM)	0.29 ± 0.00	0.16 ± 0.10*	1.0 ± 0.0	0.53 ± 0.00 *
TRAP (μM)	24 ± 2	12 ± 2 *	35 ± 1	20 ± 1 *
PAR1AP (μM)	3.9 ± 1.1	2.8 ± 0.1 *	10 ± 1	4.1 ± 0.1 *
PAR4AP (μM)	60 ± 2	46 ± 3 *	70 ± 2	59 ± 3 *

Data are mean ± SEM, n = 7. * $p<0.05$ versus control (without leukocytes)

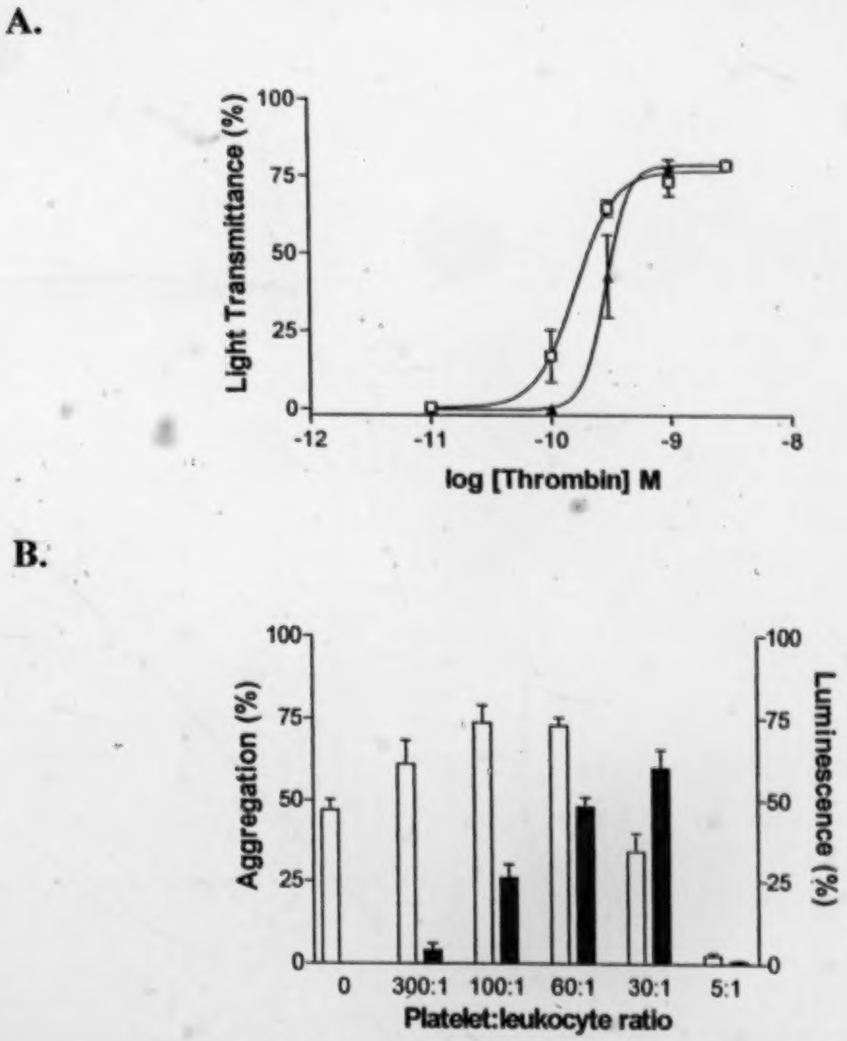


Figure 24. Leukocytes modify platelet aggregation induced by PAR agonists. (A) Washed platelets and leukocytes were coincubated at a physiological ratio 60:1 and thrombin (0.1-3 nM) was added to induce aggregation. Concentration-response curves were generated in the presence (square) or absence (triangle) of leukocytes. (B) Leukocytes induce a biphasic regulation of platelet aggregation and respiratory burst, as measured by chemiluminescence. EC_{50} of thrombin (0.3 nM) was added to stimulate aggregation; aggregation (open bars), luminol-enhanced luminescence (closed bars). Data are mean ± SEM, n = 8.

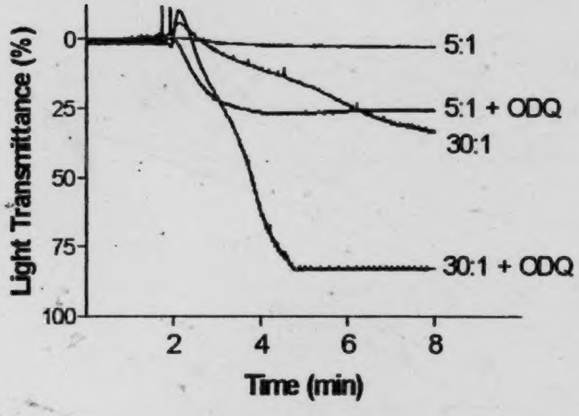


Figure 25. Reversal of inhibitory effects of high concentrations of leukocytes on platelet aggregation with ODQ. Platelet-leukocyte suspension (at ratio 5:1 or 30:1) was preincubated with ODQ (1 µM) for 2 minutes, and the aggregation was initiated by 0.3 nM thrombin. Superimposed traces are representative of 5 independent experiments.

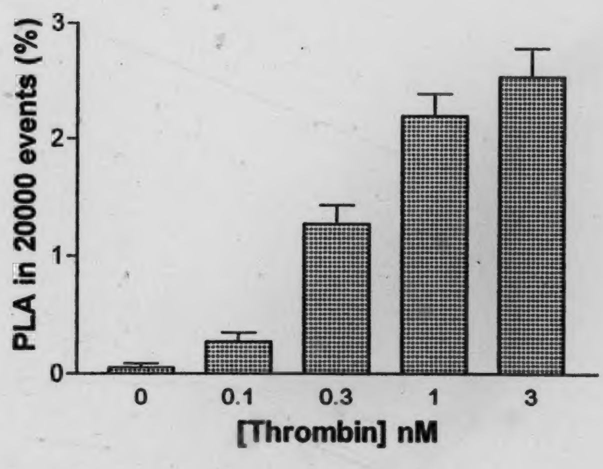


Figure 26. Flow cytometry analysis of PLA. This was induced by the addition of thrombin (0.1-3 nM). Data are mean ± SEM, n = 5.

Microparticle formation and P-selectin expression in platelets during PAR-agonist stimulation

Since the release of microparticles (MP) and the expression of P-selectin from activated platelets involve in the formation of PLA, I used flow cytometry to measure these phenomena. Submaximal concentrations of PAR agonists (Thrombin: 1 nM, TRAP: 35 μM, PAR1AP: 10 μM, PAR4AP: 70 μM) were used to stimulate the platelet release of MP and P-selectin expression. Figure 27 shows that PAR agonist-stimulation led to increased (by 35-50%) generation of MP. Interestingly, TRAP-induced MP formation was significantly lower compared with those induced by other PAR agonists (Figure 27).

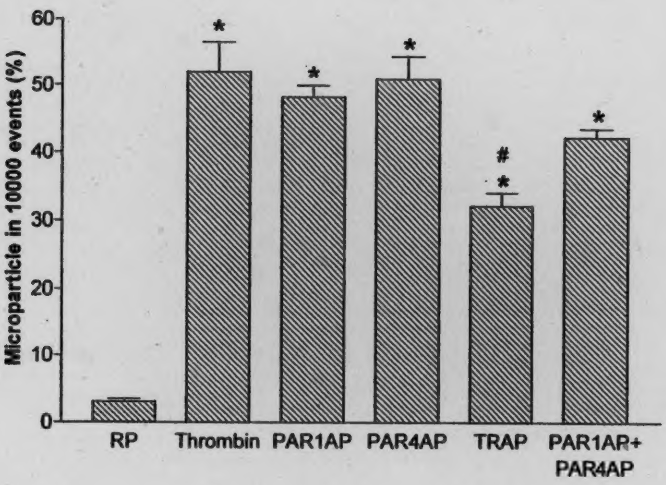


Figure 27. Flow cytometry analysis of microparticle formation during PAR agonist-stimulation. Platelets were stimulated with the submaximal concentrations (EC_{95}) of PAR agonists (shown on Table 5). Data are mean ± SEM, n = 7. * $p<0.001$ treatments versus resting platelets, RP. # $p<0.05$ TRAP versus other PAR agonists.

Stimulation of platelets with PAR agonists resulted in a significant increase in P-selectin expression by 10-12-fold, compared with control (Figure 28). Interestingly, the submaximal concentration of collagen (4 μg/mL) increased P-selectin expression only by 2.5-fold.

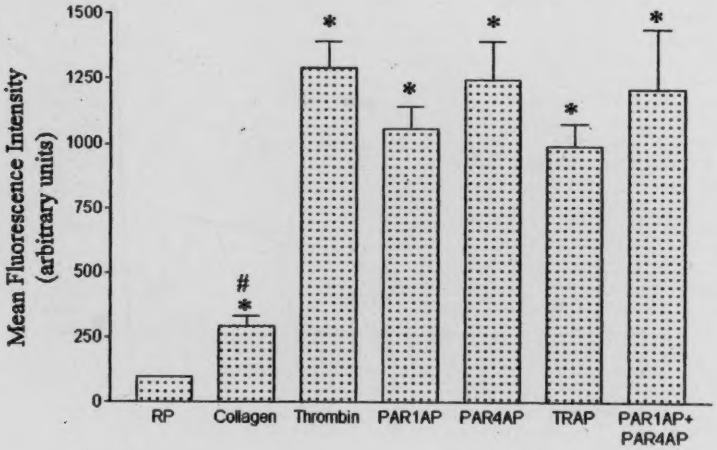


Figure 28. Flow cytometry analysis of P-selectin expression during platelet activation. Washed platelets were stimulated with EC_{95} of PAR agonists (Table 5) or 4 μg/mL of collagen. Data are mean ± SEM, n = 3-8. * $p<0.001$ treatments versus resting platelets, RP. # $p<0.001$ Collagen versus PAR agonists (ANOVA).

Microscopy of Platelet-leukocyte interactions

Figure 29A and B show PLA, as observed in phase-contrast microscopy, in control and thrombin-stimulated platelets.

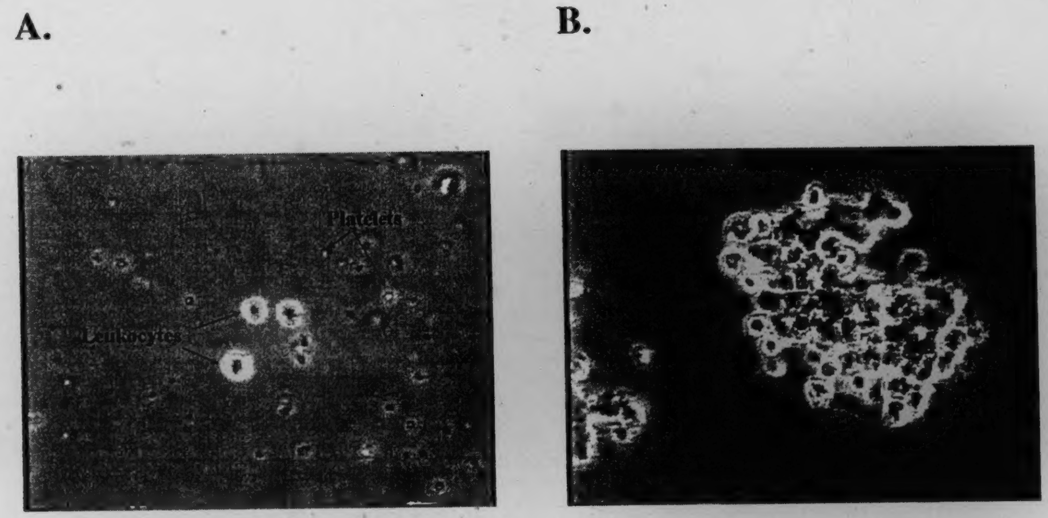


Figure 29. Microscopy showing platelet-leukocyte interactions (A) in resting sample or (B) during cell stimulation with 0.3 nM thrombin. Representative slides from 3 independent experiments (x800).

Effects of prostacyclin and nitric oxide on platelet-leukocyte interactions induced by PAR agonists

Prostacyclin or GSNO inhibited PAR agonist-induced platelet-leukocyte aggregation in a concentration-dependent manner, as measured by light aggregometry (Figure 30A and B). Figure 30C shows the synergistic effects of two inhibitors used to suppress the aggregation induced by thrombin. Similar results were obtained with other PAR agonists; for instance, PAR1AP (4 μM)-induced platelet-leukocyte aggregation was abolished in the presence of GSNO and PGI_2.

Prostacyclin and GSNO were also effective in reducing PLA induced by PAR agonists, as measured by flow cytometry (Figure 31).

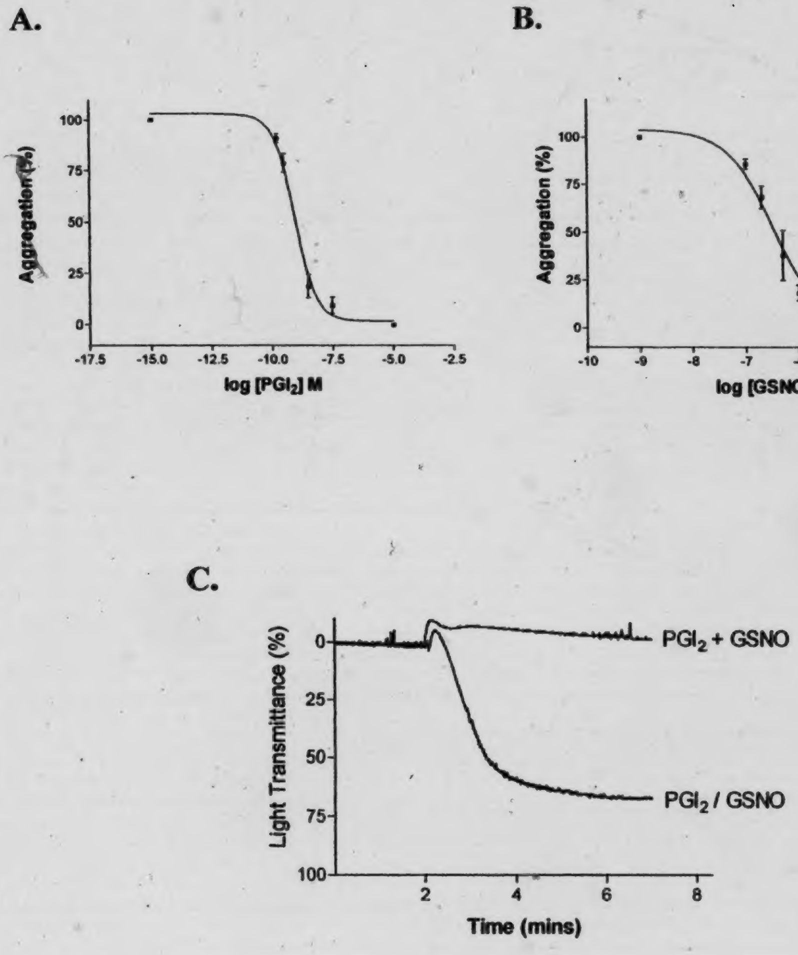


Figure 30. Effects of PGI$_2$ and GSNO on platelet-leukocyte aggregation, as measured by light aggregometry. Platelet-leukocyte suspensions were preincubated with PGI$_2$ (A, 0.1-10 nM) or GSNO (B, 0.1-100 μM). Aggregation was induced by thrombin (0.5 nM). The combination of the subthreshold concentrations of PGI$_2$ (0.3 nM) and GSNO, (1 μM) showed the synergistic inhibitory effect (C, representative of 5 independent experiments).

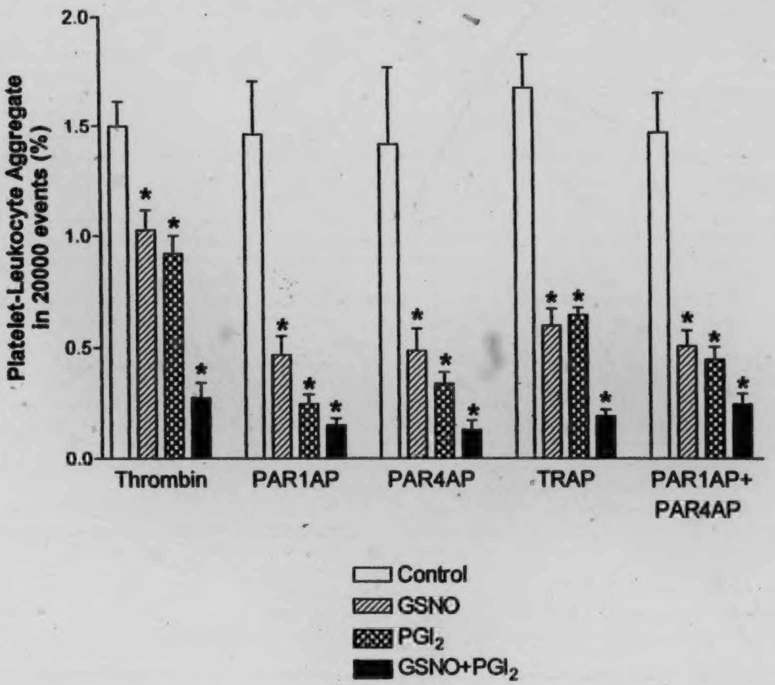


Figure 31. Flow cytometry analysis of the PLA and the effects of PGI_2 and GSNO. PLA was stimulated by EC_{50} (Table 5) of PAR agonists (control) in the presence or absence of 5 μM GSNO, 3 nM PGI_2 or both GSNO and PGI_2. Data are mean ± SEM, n = 5-9, * $p<0.05$ treatments versus control (in the absence of inhibitors).

Thrombin-induced MP formation was abolished by PGI_2 or GSNO (Figure 32). Similar results were obtained with other PAR agonists; for instance, the generation of MP induced by TRAP (35 μM) was also prevented by the presence of either inhibitor.

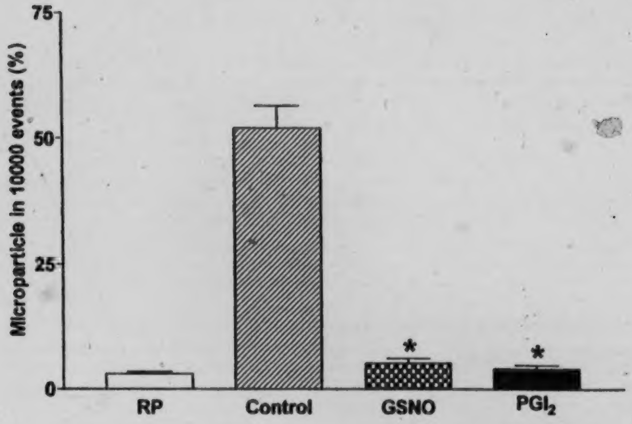


Figure 32. Flow cytometry analysis of microparticle formation during PAR agonist stimulation. Platelets stimulated with thrombin (1 nM, control) in the presence of 5 μM GSNO or 3 nM PGI_2. RP: resting platelets. * $p<0.05$ treatments versus thrombin control, in the absence of inhibitors.

PGI_2 and GSNO differentially inhibited PAR-mediated P-selectin expression (Figure 33). Indeed, GSNO reduced P-selectin expression stimulated by thrombin, TRAP, PAR1AP and PAR1AP + PAR4AP by 10-60% while PAR4AP-induced P-selectin expression was insensitive to GSNO inhibition. In contrast, PGI_2 reduced P-selectin expression by 30-70% irrespective of PAR agonist used. In all cases, except PAR1AP-mediated effects, PGI_2 was more effective than GSNO to reduce P-selectin expression (# $p<0.05$). As with PLA formation, GSNO and PGI_2 synergistically suppressed P-selectin expression stimulated by PAR agonists (Figure 33).

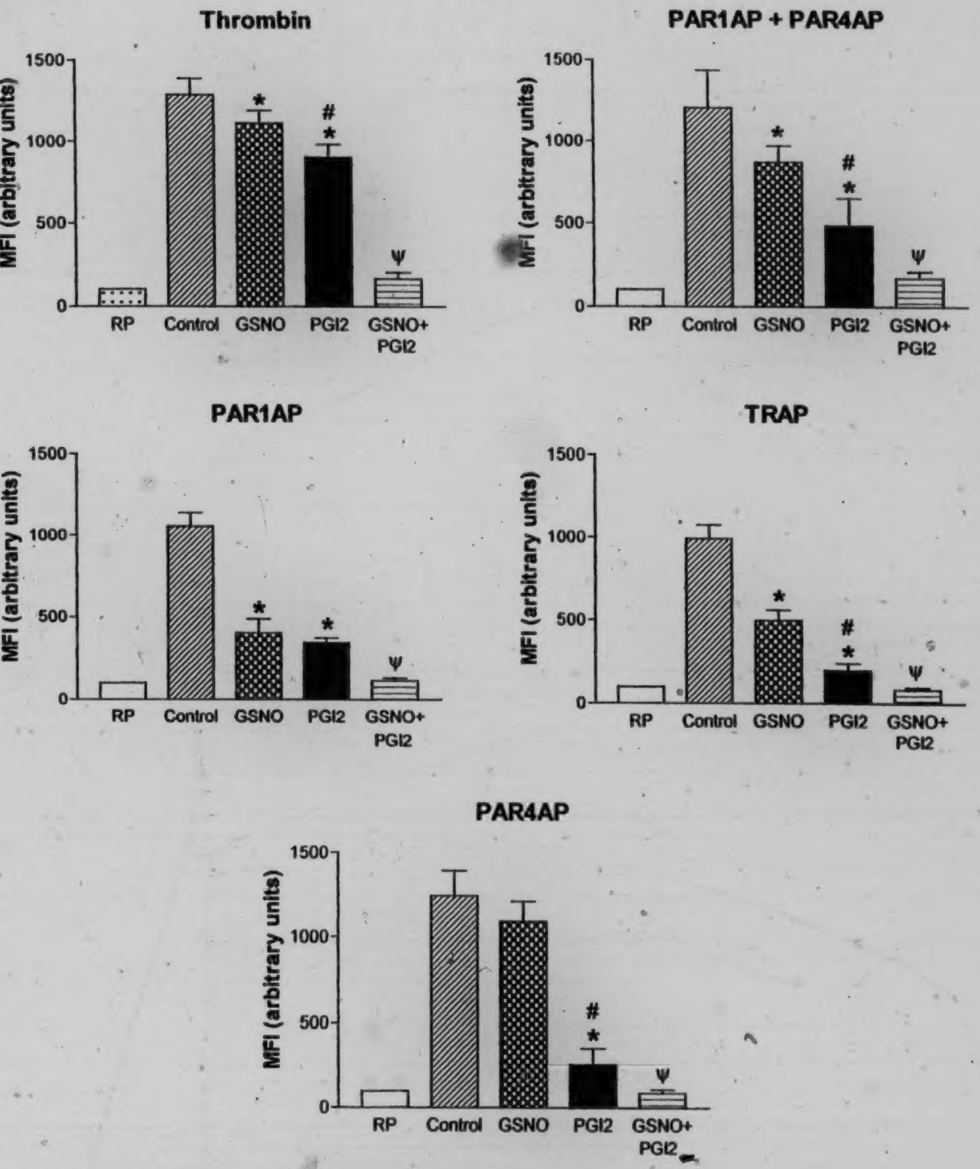


Figure 33. Flow cytometry analysis of P-selectin expression on platelets after PAR agonist-stimulation and the effects of GSNO and PGI$_2$. Washed platelets were stimulated with submaximal concentrations (EC$_{95}$, Table 5) of PAR agonists (control) in the presence of 5 μM GSNO, 3 nM PGI$_2$ or both GSNO and PGI$_2$. RP: resting platelets. MFI: mean fluorescence intensity. Data are mean ± SEM, n = 3-8. * $p<0.05$ treatments versus control (PAR agonists in the absence of inhibitors), # $p<0.05$ PGI$_2$ versus GSNO treatment, and ψ $p<0.05$ GSNO+PGI$_2$ versus PGI$_2$.

Thromboxane A_2, ADP and MMP-2 in platelet-leukocyte interactions

To study the dependence of platelet-leukocyte aggregation on activation of the TXA_2-, MMP-2- and ADP-mediated pathways of aggregation, respective inhibitors of these pathways such as aspirin, phenanthroline and apyrase were used. Light aggregometry measurements showed that all three inhibitors reduced, in a significant way, platelet-leukocyte aggregation (Figure 34). PAR4AP-induced aggregation was highly susceptible to inhibition with the three inhibitors. Aspirin exerted approximately 30% inhibition of thrombin-, TRAP-, PAR1AP- and PAR1AP + PAR4AP-induced aggregation, as compared to controls. Thrombin-induced aggregation was more sensitive to inhibition with apyrase than aggregation induced by other PAR agonists. Phenanthroline inhibited PAR agonist-induced aggregation by 70-95%.

ASA, apyrase and phenanthroline exerted differential effects on PLA, as measured by flow cytometry (Figure 35). ASA exerted inhibition only of TRAP- and PAR1AP + PAR4AP-induced PLA formation. Apyrase was effective against PLA formation induced by all tested agonists, with the exception of PAR1AP. Interestingly, phenanthroline was the only inhibitor significantly reducing PLA induced by all PAR agonists.

Figure 36A-C shows microscopical examination of PLA during thrombin stimulation in the presence of aspirin, apyrase or phenanthroline.

ASA, phenanthroline and apyrase reduced the formation of microparticles (Figure 37) and the expression of P-selectin (Figure 38) induced by PAR agonists.

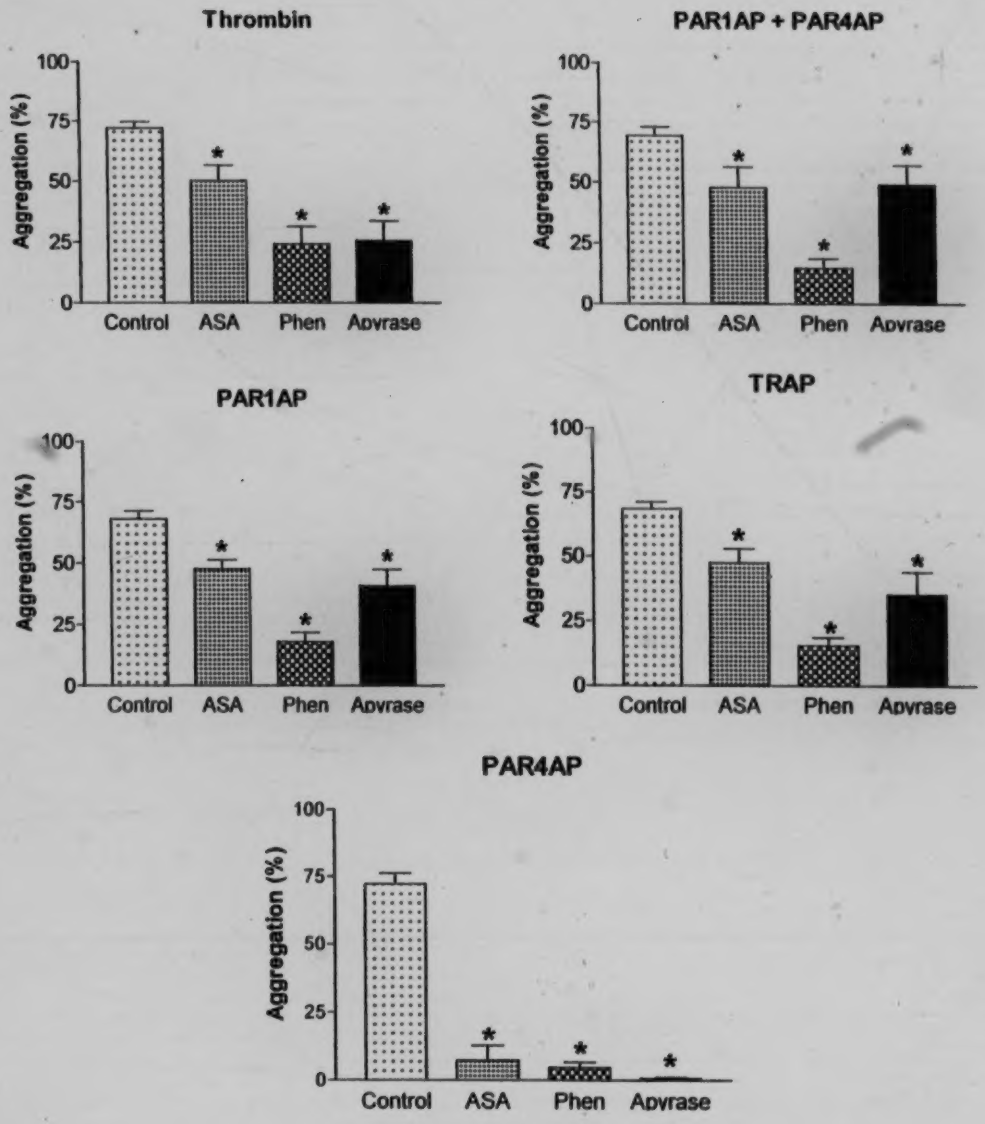


Figure 34. Effects of aspirin, phenanthroline and apyrase on platelet-leukocyte aggregation, as measured by optical aggregometry. Submaximal concentrations of PAR agonists (EC_{95}, shown on Table 5) were used to initiate aggregation in the presence of aspirin (ASA, 300 μM), phenanthroline (Phen, 100 μM) and apyrase (Apy, 300 μg/mL). Data are mean ± SEM, n = 7-16, * $p<0.05$ treatments versus control.

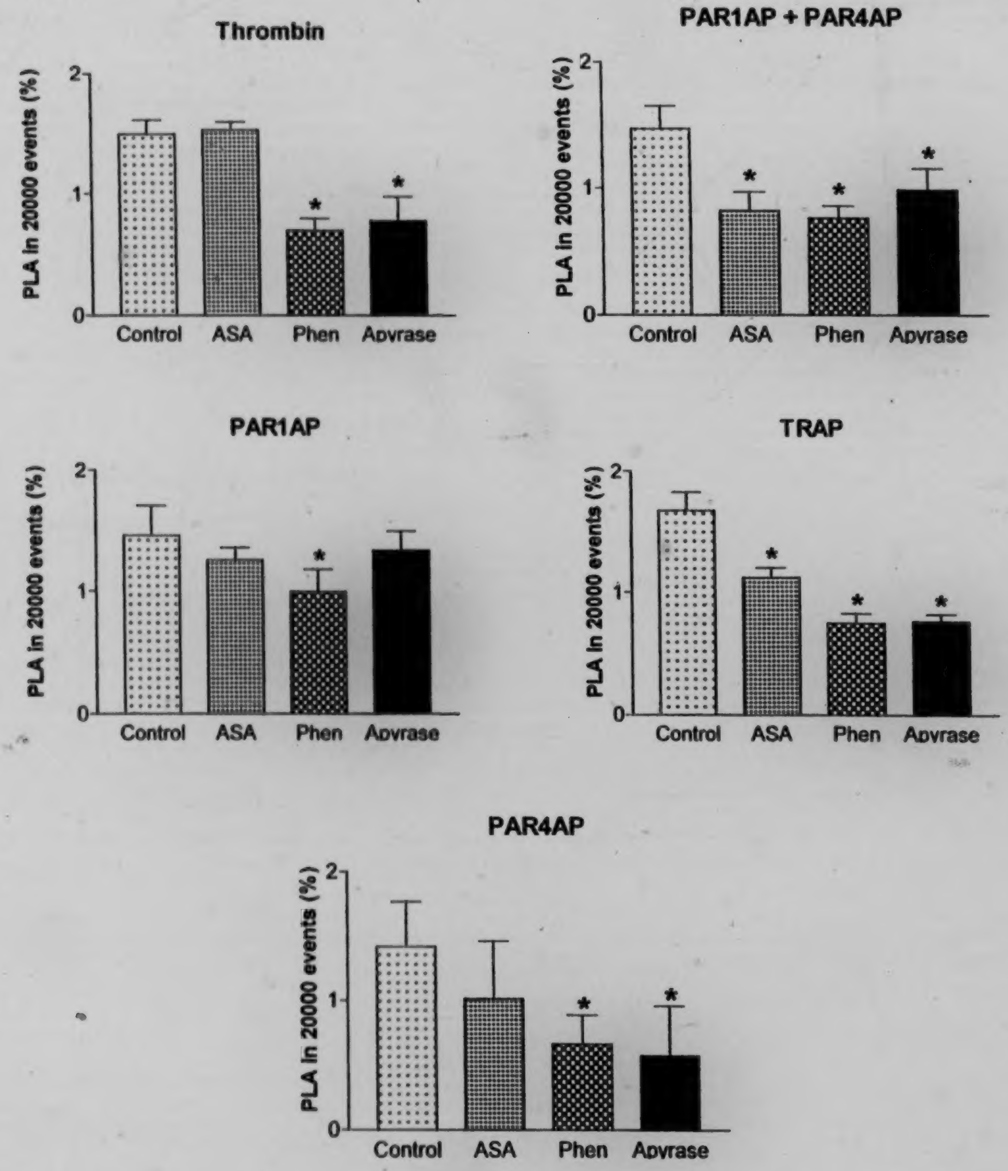


Figure 35. Effects of aspirin, phenanthroline and apyrase on PLA as measured by flow cytometry. PLA was stimulated by the submaximal concentrations of PAR agonists (EC_{95}, shown on Table 5, Control) in the presence of aspirin (ASA, 300 μM), phenanthroline (Phen, 100 μM) or apyrase (Apy, 300 μg/mL). Data are mean ± SEM, n = 5-9, * $p<0.05$ treatments versus control.

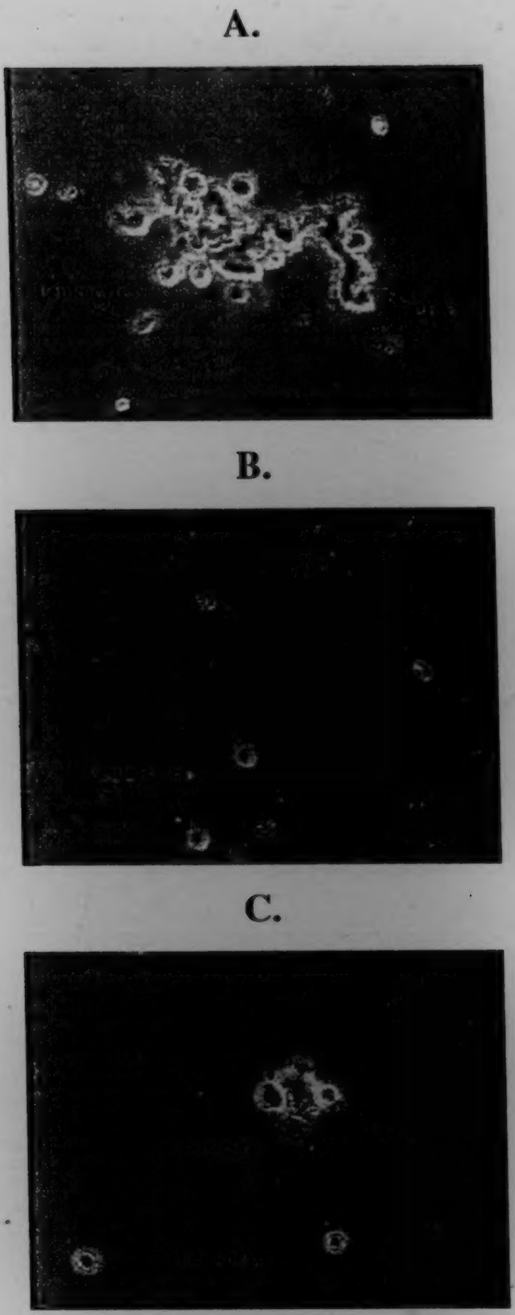


Figure 36. Microscopy examination showing inhibition of PLA induced by 0.3 nM thrombin in the presence of (A) 300 μM aspirin, (B) 100 μM phenanthroline or (C) 300 μg/mL apyrase. Representative slides from 3 independent experiments (x 800).

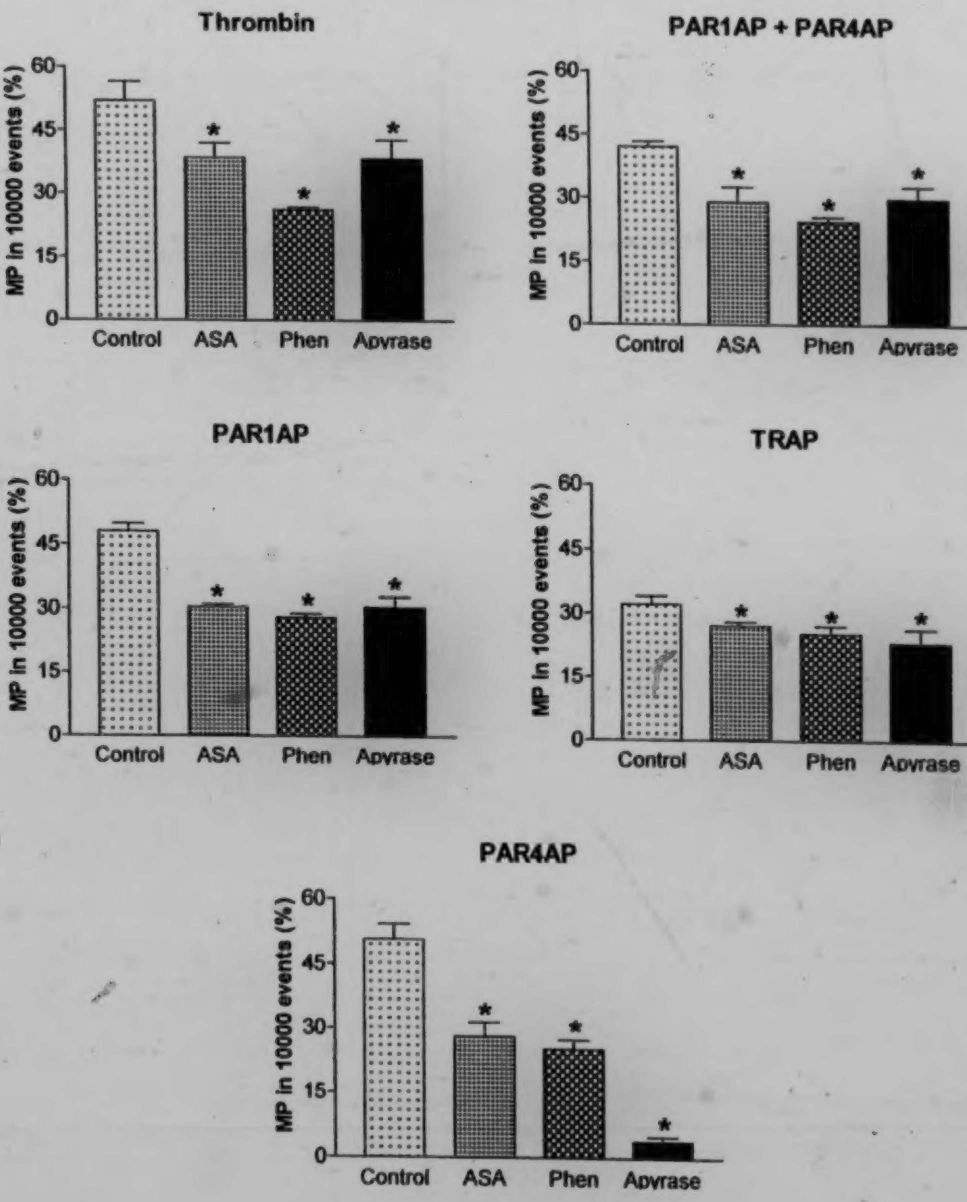


Figure 37. Effects of aspirin, phenanthroline and apyrase on microparticle formation. Platelets were stimulated with the submaximal concentrations of PAR agonists (EC_{95}, shown on Table 5, Control) in the presence of aspirin (ASA, 300 μM), phenanthroline (Phen, 100 μM) or apyrase (Apy, 300 μg/mL). Data are mean ± SEM, n = 5-9. * $p<0.05$ treatments versus control.

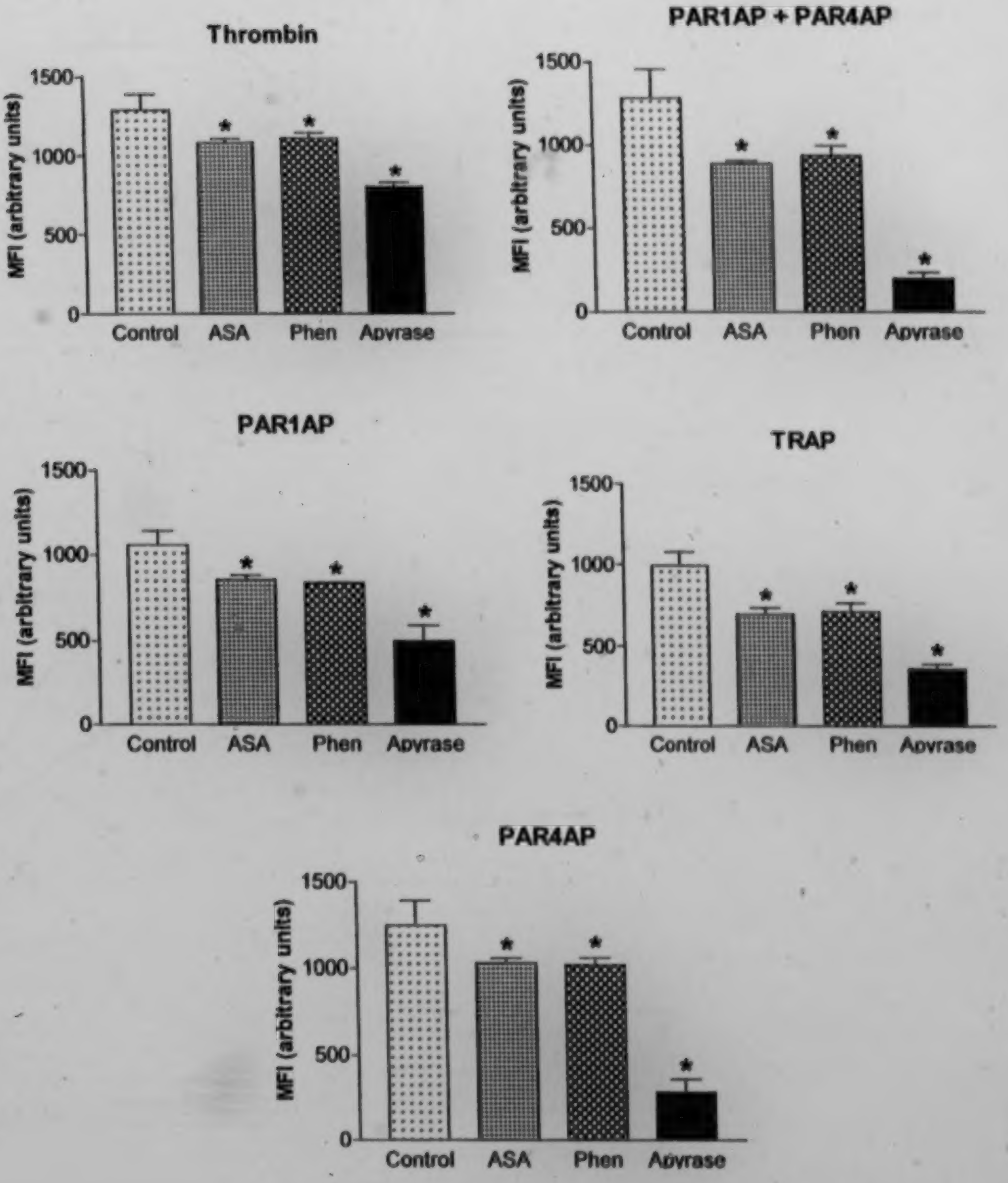


Figure 38. Effects of aspirin, phenanthroline and apyrase on P-selectin expression. Washed platelets were activated by the submaximal concentrations of PAR agonists (EC_{95}, shown on Table 5, Control) in the presence of aspirin (ASA, 300 μM), phenanthroline (Phen, 100 μM) and apyrase (Apy, 300 μg/mL). MFI: mean fluorescence intensity. Data are mean ± SEM, n = 5-10. * $p<0.05$ treatments versus control.

Matrix metalloproteinases in platelet-leukocyte interactions

From the data of the flow cytometry analysis and the microscopical examination, phenanthroline was shown to be the most effective inhibitor compared with aspirin and apyrase in suppressing the PLA formation, implicating the significant role of MMPs in platelet-leukocyte interactions. Therefore, I performed additional experiments to confirm the importance of MMPs. Figure 39A-D shows Western blot analysis of MMP-1, MMP-2, MMP-3 and MMP-9 in platelets and/or leukocytes stimulated with PAR agonists. Following PAR-induced aggregation, the MMP expression was assayed in the particulate (pellet) and soluble (releasate) fractions of platelets and leukocytes. PAR agonists stimulated the translocation of active MMP-1, -2, -3 and –9 from the pellet to the releasate ($n = 6$, $p<0.05$). The levels of latent proteins were not significantly altered by PAR-agonists (data not shown)..

Flow cytometry analysis showed the presence of MMP-1, -2, -3 and –9 on the surface of PLA during thrombin stimulation (Figure 40A-D). Similar results were obtained with other PAR agonists (data not shown).

The MMP-1 and MMP-3 activities in releasates and pellets of platelet-leukocyte incubates were analyzed by the enzyme activity assay kits (Figure 41A and B). The addition of thrombin significantly increased the levels of active MMP-1 and MMP-3 in the releasate, with a corresponding decrease in the pellet ($p<0.05$). Similar results were obtained with other PAR agonists (data not shown).

The activities of MMP-2 and MMP-9 were analyzed by zymography (Figure 42). Thrombin significantly increased the release of pro-MMP-2 and MMP-2 into platelet releasate. Since zymography did not resolve well pro-MMP-9 and MMP-9, both pro and active forms of MMP-9 were analyzed together as the total MMP-9, and this was significantly increased following thrombin stimulation. The activities of MMP-2 and MMP-9 in pellets were not altered by thrombin ($p>0.05$, data not shown).

A. **MMP-1**

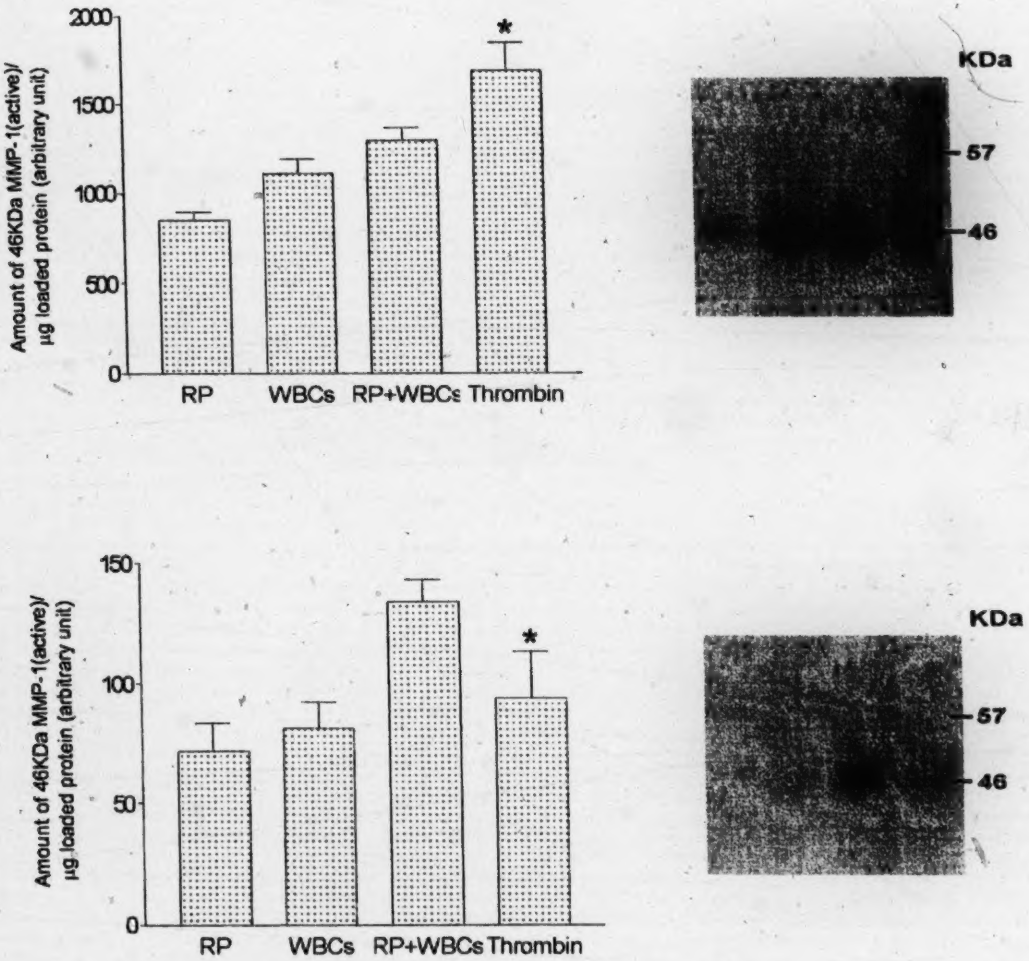
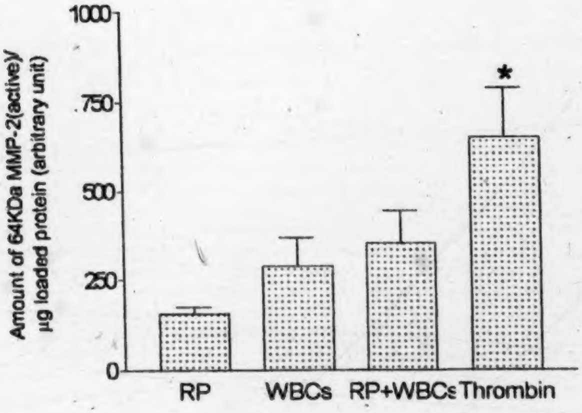


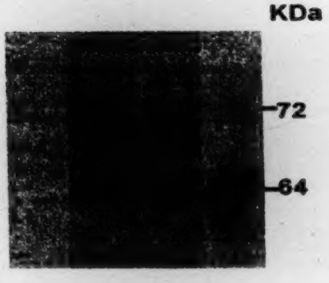
Figure 39A-D. Western blot analysis of MMPs in platelet-leukocyte incubates. A-D, the densitometric analysis of MMP-1 (A), MMP-2 (B), MMP-3 (C) and MMP-9 (D) in supernatants (upper panel) and pellets (lower panel) of platelet-leukocyte incubates. RP: resting platelets, WBCs: white blood cells, RP+WBCs: resting platelets and white blood cells (60:1 ratio), Thrombin: RP+WBCs stimulated with thrombin (0.5 nM). Data are mean ± SEM, n = 6. Inserts showed a representative blotting of 6 independent experiments. * $p<0.05$ thrombin versus RP+WBCs.

B.

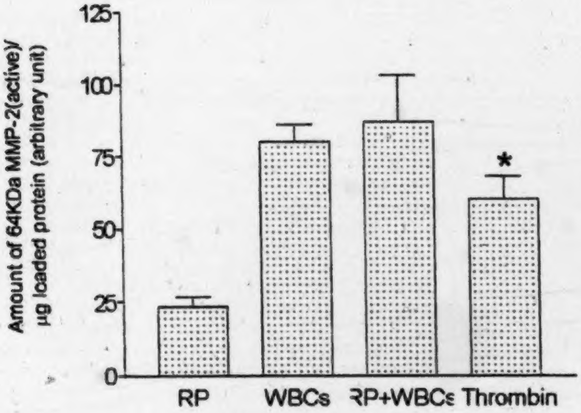
MMP-2



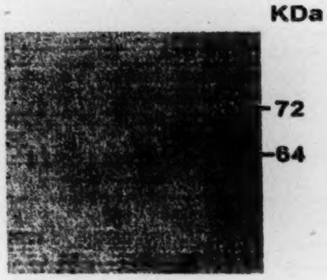
Amount of 64KDa MMP-2(active)/ µg loaded protein (arbitrary unit)
1000
750
500
250
0
*
RP
WBCs
RP+WBCs
Thrombin



KDa
72
64

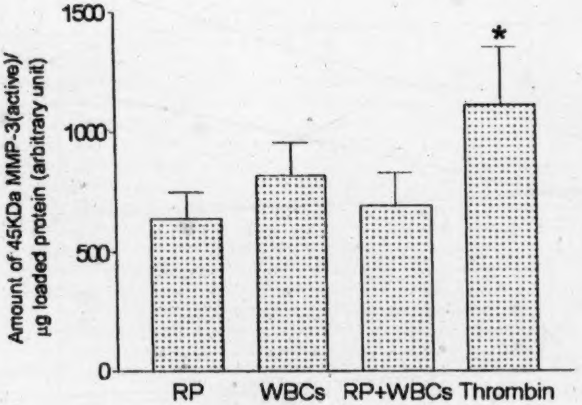


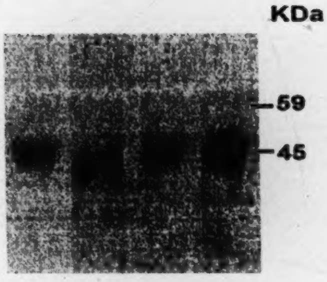
Amount of 64KDa MMP-2(active)/ µg loaded protein (arbitrary unit)
125
100
75
50
25
0
*
RP
WBCs
RP+WBCs
Thrombin

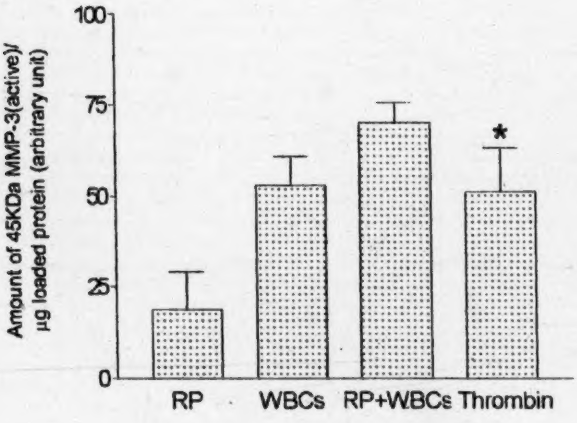


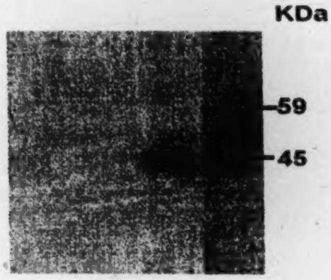
KDa
72
64

C. **MMP-3**


Amount of 45KDa MMP-3(active)/
µg loaded protein (arbitrary unit)
1500
1000
500
0
*
RP
WBCs
RP+WBCs
Thrombin

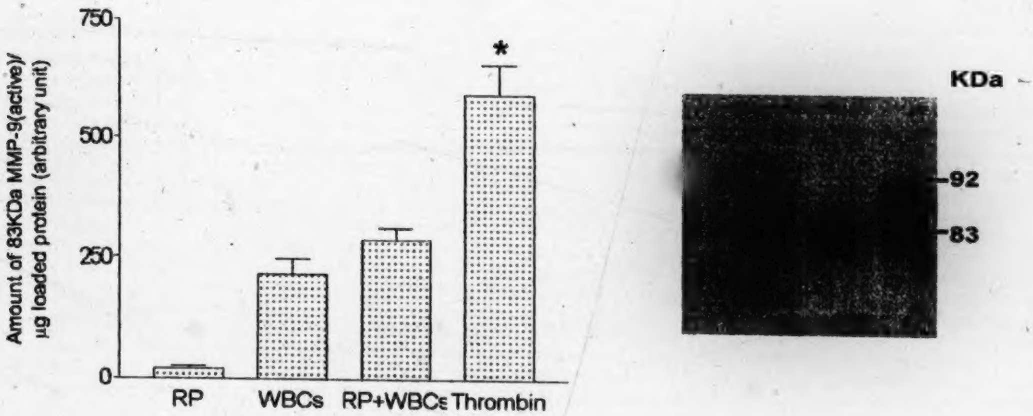

KDa
59
45

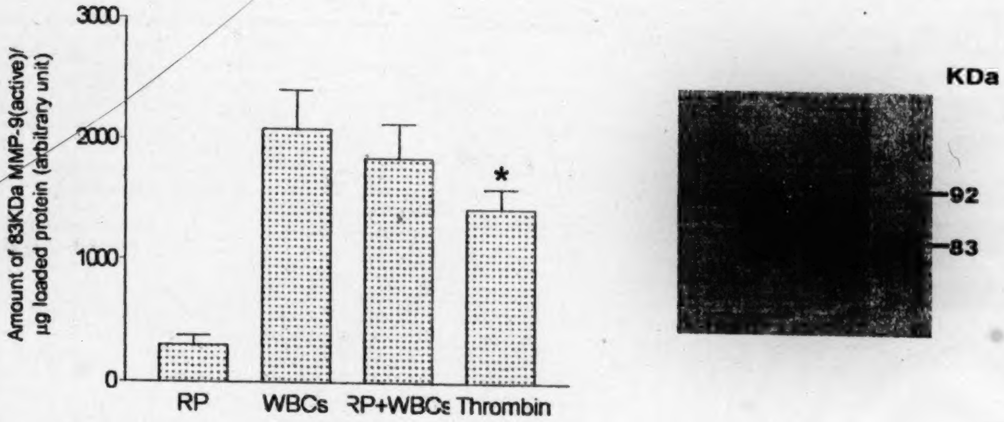

Amount of 45KDa MMP-3(active)/
µg loaded protein (arbitrary unit)
100
75
50
25
0
*
RP
WBCs
RP+WBCs
Thrombin


KDa
59
45

D.

MMP-9


750
500
250
0
Amount of 83KDa MMP-9(active)/
µg loaded protein (arbitrary unit)
*
RP
WBCs
RP+WBCs
Thrombin
KDa
92
83


3000
2000
1000
0
Amount of 83KDa MMP-9(active)/
µg loaded protein (arbitrary unit)
*
RP
WBCs
RP+WBCs
Thrombin
KDa
92
83

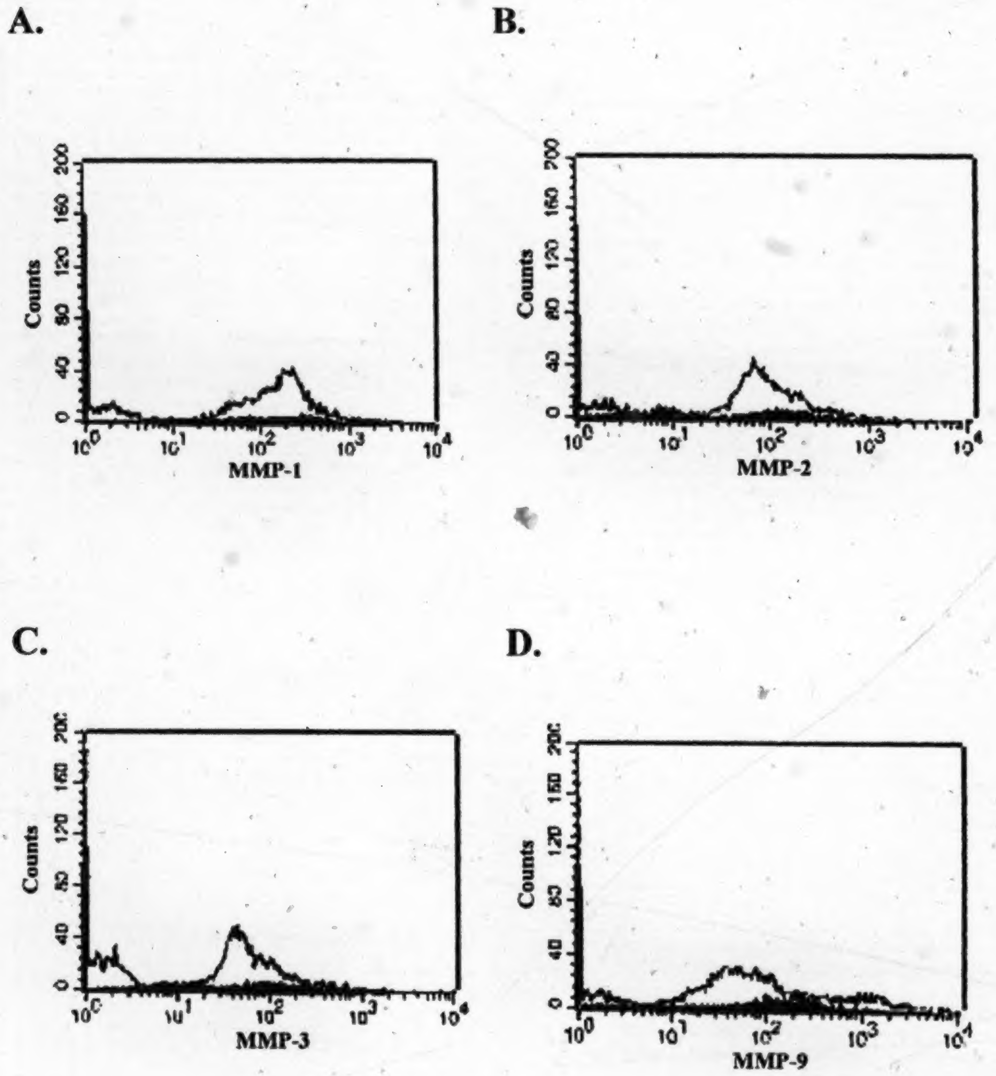


Figure 40. Flow cytometry of MMPs on the surface of PLA. Resting (RP) or thrombin (0.3 nM) stimulated platelet-leukocyte suspensions (60:1 ratio) were treated as described in Materials and Methods. A-D, fluorescence intensities when resting (shaded line) and thrombin-stimulated (solid line) samples were treated with specific MMP-1 (A), MMP-2 (B), MMP-3 (C) and MMP-9 (D) monoclonal antibodies. Results are representative of 5 independent experiments.

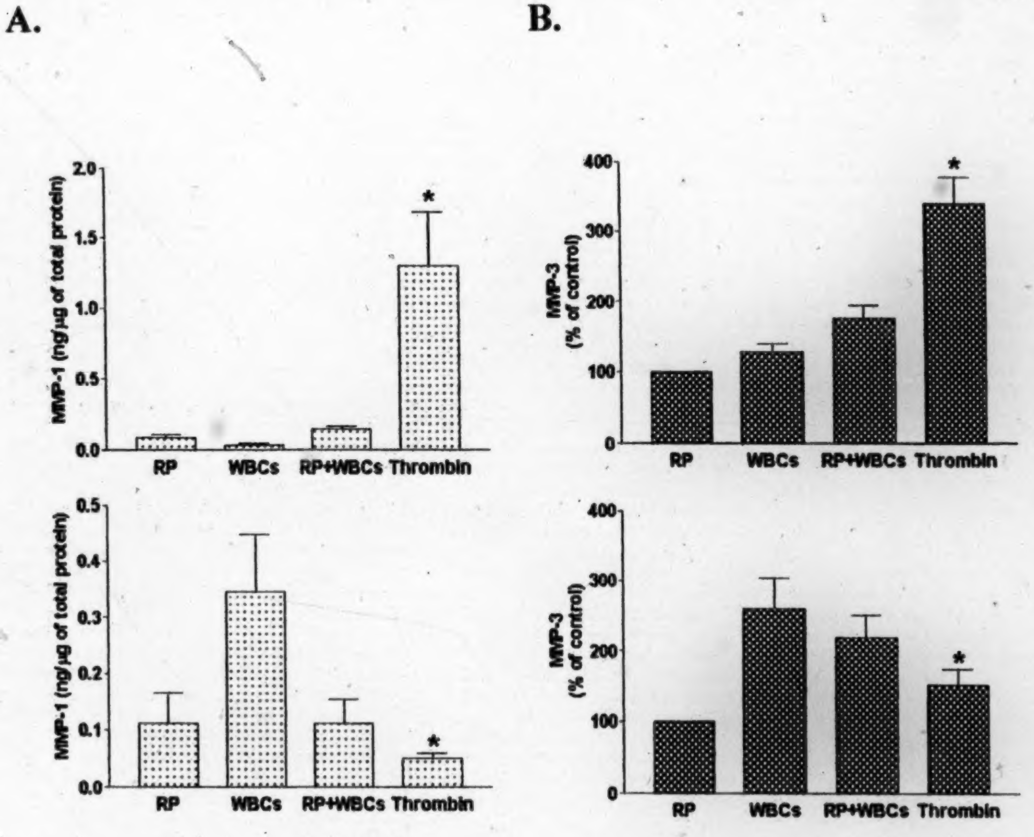


Figure 41. MMP-1 and MMP-3 activity in platelet-leukocyte incubates. The MMP-1 (A) and MMP-3 (B) activity of the releasate (upper panel) and the pellets (lower panel) of platelet-leukocyte suspension (60:1 ratio). The MMP-3 activity was normalized to control (RP) levels. RP: resting platelets, WBCs: white blood cells, RP+WBCs: resting platelets and white blood cells, Thrombin: RP+WBCs stimulated with thrombin. Data are mean ± SEM, n = 3-6. * $p<0.05$ thrombin versus RP+WBCs.

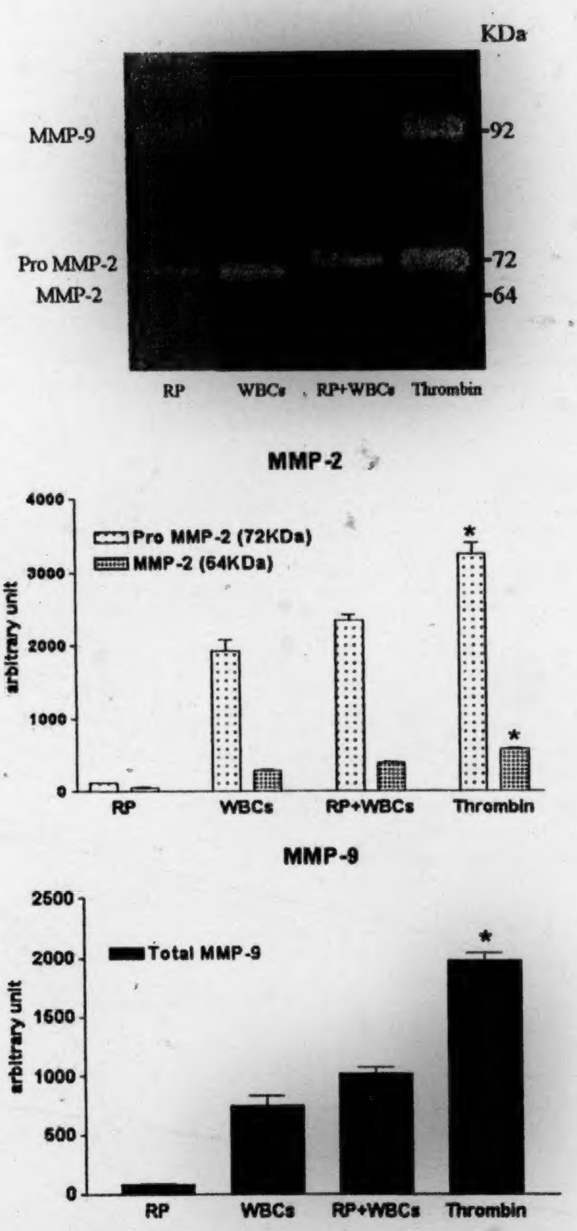


Figure 42. The activity of MMP-2 and MMP-9 in the releasate of platelet-leukocyte incubates. The top panel is the representative zymograms. Bar graphs are the densitometric analysis of MMP-2 and MMP-9 activity. RP: resting platelets, WBCs: white blood cells, RP+WBCs: resting platelets and white blood cells (60:1 ratio), Thrombin: RP+WBCs stimulated with thrombin. Data are mean ± SEM, n = 8. * $p<0.05$ thrombin versus RP+WBCs.

Recombinant human MMP-2 and MMP-3, supplied as a mixture of zymogen and active enzyme, potentiated platelet-leukocyte aggregation induced by thrombin (Figure 43). Recombined human MMP-1 and MMP-9, containing zymogen mainly, did not influence aggregation (n = 5). Similar results were obtained with other PAR agonists.

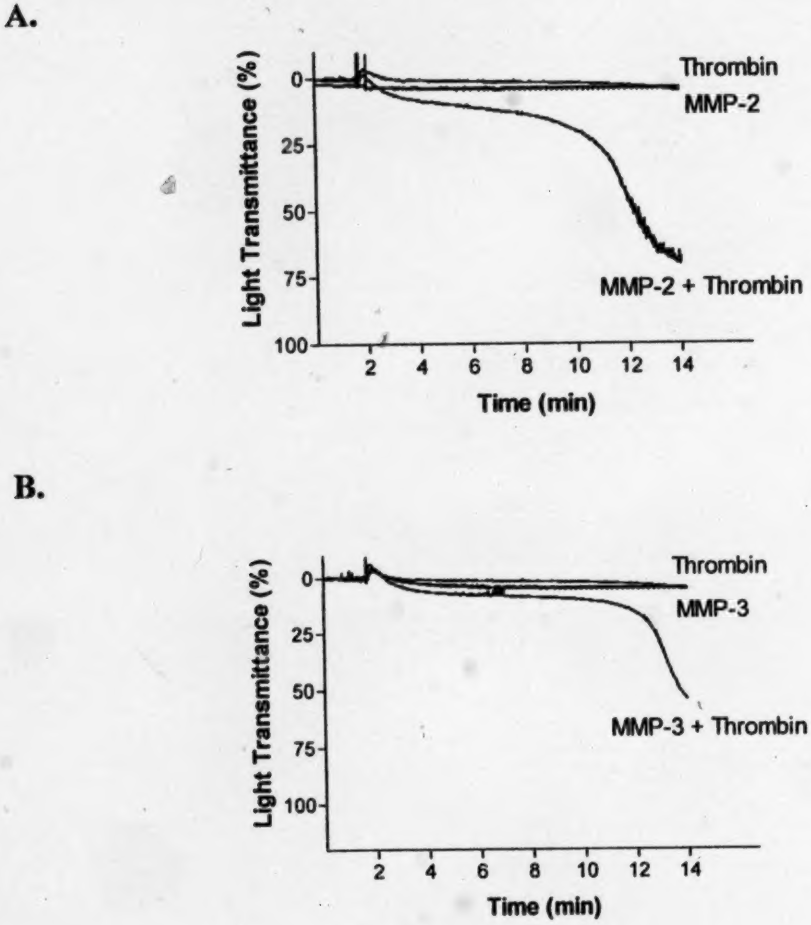


Figure 43. Effects of human recombinant MMPs on platelet-leukocyte aggregation stimulated with thrombin. Platelet-leukocyte suspensions (60:1 ratio) were preincubated with (A) MMP-2 (1 μg) or (B) MMP-3 (2.6 μg) before stimulation with the subthreshold concentration of thrombin (0.1 nM). Results are representative of 5 independent experiments.

Neutralizing monoclonal antibodies against latent and active forms of MMP-1, -2, -3 inhibited platelet-leukocyte aggregation induced by thrombin in a concentration-dependent manner (Figure 44). Anti-MMP-9 antibodies regulated the aggregation in a biphasic manner. Low concentrations (0.5-1 μg/mL) of this antibody potentiated (by 3-12%), while high (5-10 μg/mL) inhibited (by 30%) the aggregation (Figure 44).

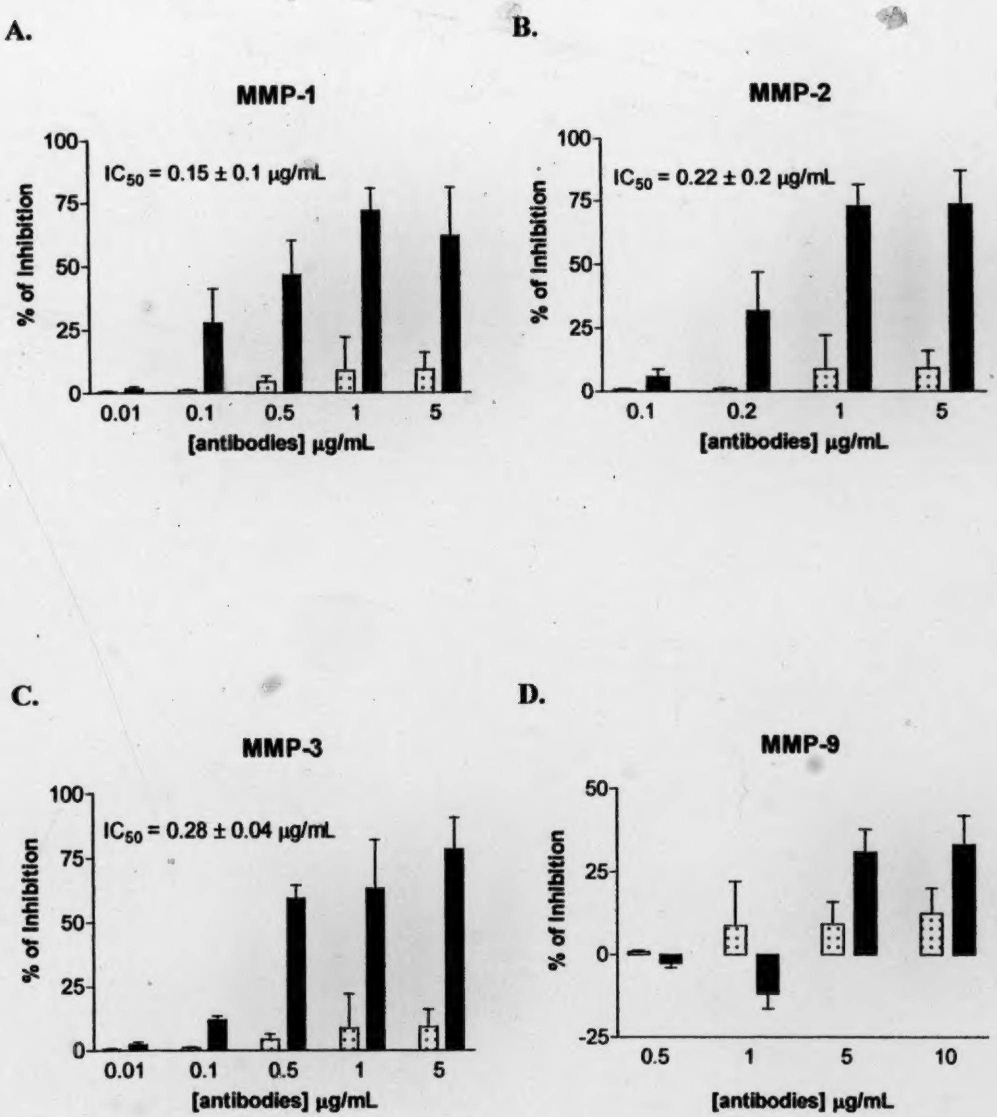


Figure 44. Effects of neutralizing monoclonal antibodies against MMPs on platelet-leukocyte aggregation. Platelet-leukocyte suspensions (60:1 ratio) was preincubated with monoclonal antibodies (closed bars) against (A) MMP-1, (B) MMP-2, (C) MMP-3, (D) MMP-9 or rabbit IgG control (dotted bars) before thrombin stimulation (0.3 nM). Bars are mean ± SEM, n = 6.

Effects of CPB on Platelet and Leukocyte Functions

In order to investigate the influence of the CPB procedure on platelet and leukocyte functions, activation markers of these cells were analyzed before initiation and after termination of CPB. CPB led to a significant reduction of platelet (19 ± 2 %) and increase in leukocyte (97 ± 14 %) numbers (Figure 45). The levels of GPIb were downregulated by 13 ± 1 %, while P-selectin was increased by 24 ± 3 %. Total and activated GPIIb/IIIa were reduced by 24 ± 2 and 11 ± 1 %, respectively (Figure 46). There was a significant reduction of collagen- and TRAP-induced aggregation (collagen EC_{50} changed from 1.1 ± 0.0 to 1.7 ± 0.1 μg/mL and TRAP EC_{50} changed from 5.9 ± 0.2 to 10 ± 0.1 μM). The maximal aggregatory response (E_{max}) of collagen was not changed, while the E_{max} of TRAP was depressed by 35 ± 7 % (Table 6). The concentration response curves to collagen and TRAP were right-shifted with an increase of EC_{50} as a result of CPB (Figure 47A). Mac-1 levels, the numbers of PLA and MPs were elevated by 150 ± 24, 50 ± 12 and 150 ± 27 %, respectively (Figure 46).

Effects of NO and PGI_2 on CPB-induced changes in platelet and leukocyte functions

We also investigated the effects of NO and PGI_2 (iloprost) on platelet and leukocyte function during CPB. These agents are already in clinical use and are known to inhibit platelet activation and platelet-leukocyte interactions in cardiac patients (Bernabei *et al*, 1995; Sly *et al*, 1995; Weerasinghe & Taylor, 1998). We wished to determine if the local administration (delivered directly into the oxygenator of the bypass circuit) of these drugs would preserve platelet and leukocyte functions during CPB.

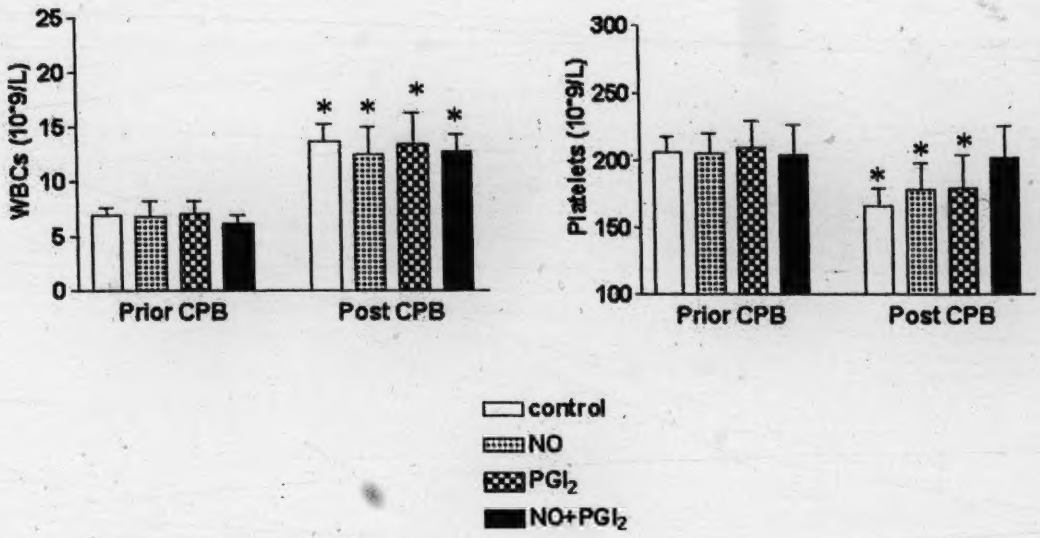
In the presence of NO, platelet count was reduced by 13 ± 2 %. This reduction was significantly less pronounced ($p<0.05$) than that in the absence of NO (18.9 ± 1.9 % in control). The NO treatment did not prevent the elevation of WBCs (84 ± 25 % increase, Figure 45 and 48). The CPB-mediated down-regulation of GPIb and total GPIIb, as well as elevation of Mac-1 level, MP and PLA formation were all attenuated by NO (Figure 46 and 49). The NO treatment also prevented CPB-induced changes in P-selectin and active GPIIb. Collagen-induced aggregation was preserved by NO such that the treatment prevented CPB-induced changes in EC_{50} value for this agonist (Figure 47B and Table 6). The EC_{50} value for TRAP-induced aggregation was unchanged, while E_{max} was reduced by 29 ± 4 % after the surgery (Table 6). However, this value was significantly ($p<0.05$) lower than that obtained in the absence of NO (Figure 47B and 50).

In the presence of iloprost, platelet numbers were reduced by 14 ± 2 %, and this decrease was less pronounced ($p<0.05$) than that in the absence of this drug. Iloprost had no effect on leukocyte numbers such that WBCs remained elevated by 92 ± 25 % (Figure 45 and 48) after CPB. CPB-induced changes in GPIb, active GPIIb, P-selectin, MP and PLA were attenuated by iloprost administration (Figure 46). In contrast, iloprost inhibited downregulation of total GPIIb (by 11 ± 1 %) and upregulated expression of Mac-1 by 299 ± 30 % (Figure 49). The iloprost treatment resulted in preservation of collagen-induced aggregation (Figure 47C and Table 6). In contrast to collagen, the sensitivity of platelets to aggregation with TRAP was reduced, with 121 ± 22 % increase in EC_{50}, and 7.7 ± 0.6 % decrease in E_{max} (Table 6).

The combined NO+PGI_2 treatment did not prevent the elevation of WBC count; however, it maintained the platelet count throughout the surgery and this effect could not be observed in other groups (Figure 45). The CABG surgery exerted no significant effects on GPIb, P-selection expression and MP formation in the presence of NO and

PGI_2 (Figure 46). The combined treatment attenuated the reduction of total GPIIb and Mac-1 expression (Figure 49). However, this treatment resulted in a reduction of active GPIIb and elevation of PLA (Figure 49). The EC_{50} for collagen-induced aggregation was reduced by 12 ± 0.4 %, and this was in contrast to control EC_{50} (51 % increase). The EC_{50} for TRAP was increased by 29 ± 0.5 %, significantly less than the control value (75 % increase). The E_{max} for both collagen- and TRAP-induced aggregatory responses were not changed significantly in the presence of PGI_2 and NO (Table 6 and Figure 47D). Table 7 shows the percent changes of cAMP and cGMP measured by enzyme immunoassay in blood cells and cell-free plasma samples (the pre heparin-values in each cases were set to be control values as 100%). NO, PGI_2 or the combination of both did not significantly influence, the levels of cAMP or cGMP ($p>0.05$).

Figure 45. Effects of NO and PGI$_2$ on platelet and leukocyte numbers during CABG surgery.



Data are mean ± SEM, n = 5-18. * $p<0.05$ post CPB versus prior CPB.

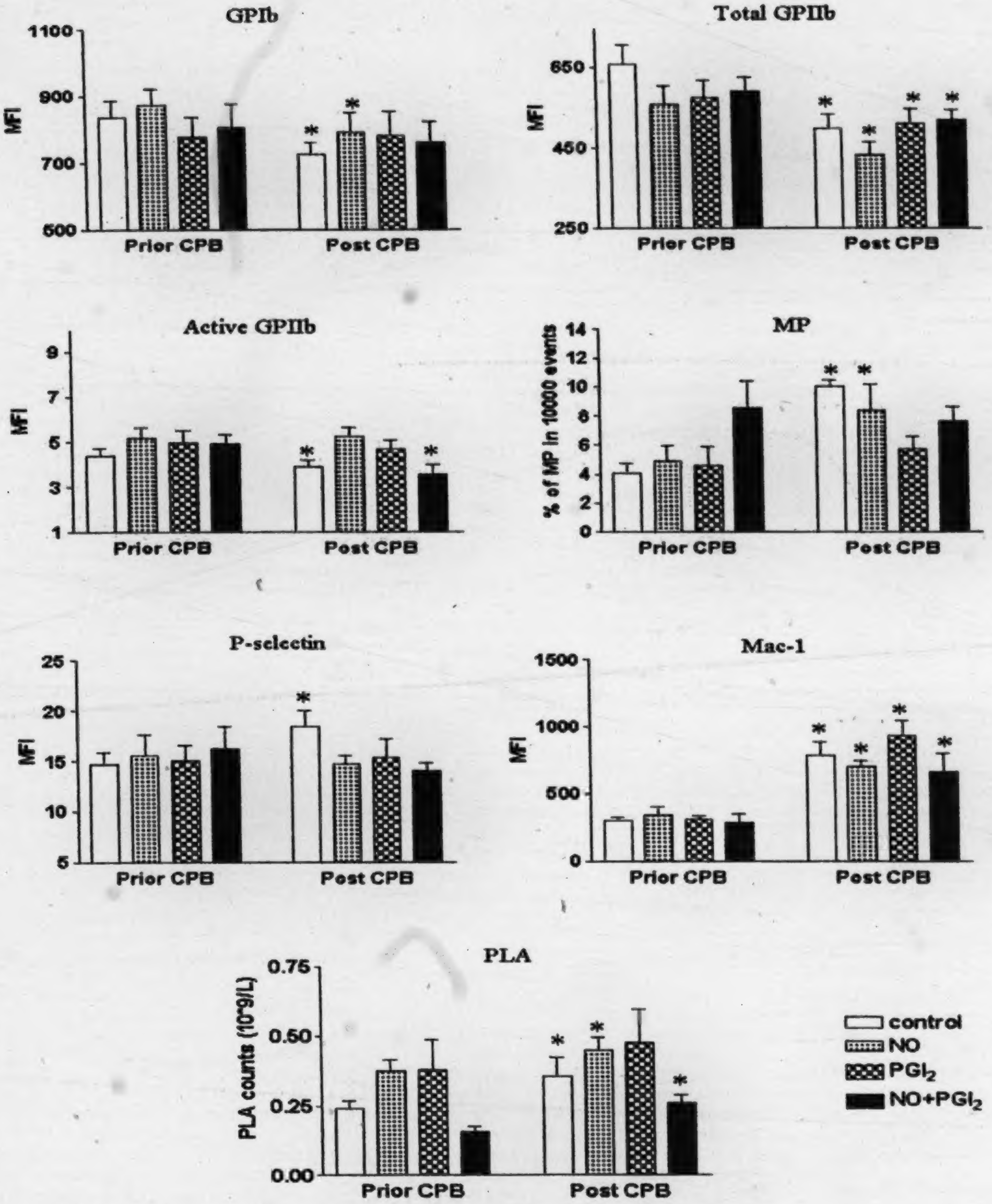


Figure 46. Effects of NO and PGI_2 on the expression of major platelet receptors, leukocyte integrin, MP formation and PLA during CABG surgery. Data are mean ± SEM, n = 5-18. * $p<0.05$ post CPB versus prior CPB.

Table 6. Effects of NO and PGI_2 on collagen- and TRAP-induced aggregation in whole blood.

Collagen

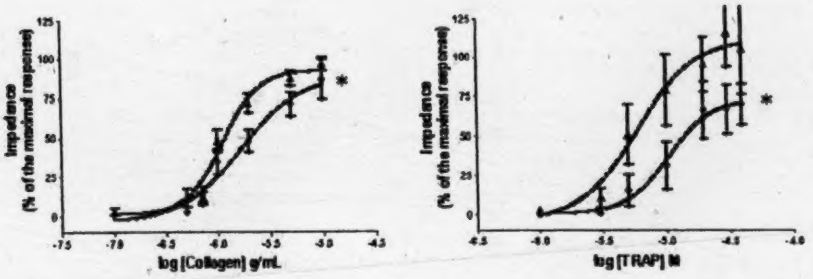
Treatment		Sample Prior CPB	Post CPB
Control	EC_{50}	1.1 ± 0.0	1.7 ± 0.1 *
	E_{max}	92 ± 4	89 ± 13
NO	EC_{50}	1.3 ± 0.1	1.3 ± 0.2
	E_{max}	100 ± 10	100 ± 19
PGI_2	EC_{50}	1.1 ± 0.04	1.1 ± 0.0
	E_{max}	94 ± 8	88 ± 7
NO + PGI_2	EC_{50}	1.3 ± 0.0	1.1 ± 0.0 *
	E_{max}	110 ± 30	100 ± 6

TRAP

Treatment		Sample Prior CPB	Post CPB
Control	EC_{50}	5.9 ± 0.2	10 ± 0.1 *
	E_{max}	110 ± 16	73 ± 10 *
NO	EC_{50}	17 ± 2	19 ± 2
	E_{max}	140 ± 10	98 ± 11 *
PGI_2	EC_{50}	5.2 ± 0.5	11 ± 2 *
	E_{max}	100 ± 6	92 ± 5 *
NO + PGI_2	EC_{50}	7.8 ± 0.1	10 ± 0.0 *
	E_{max}	100 ± 22	100 ± 11

Collagen EC_{50} (50% of maximal aggregatory response) is expressed as μg/mL, while TRAP EC_{50} is in μM. E_{max} values are calculated as a percent of maximal aggregatory responses in the patient. Data are mean ± SEM, n = 5-18. * $p<0.05$ post CPB versus prior CPB.

A. **Control**



B. **NO treatment**

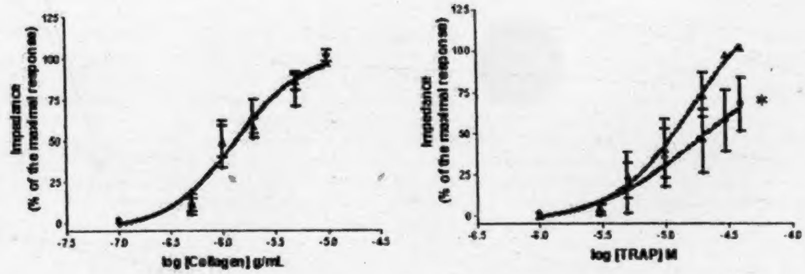
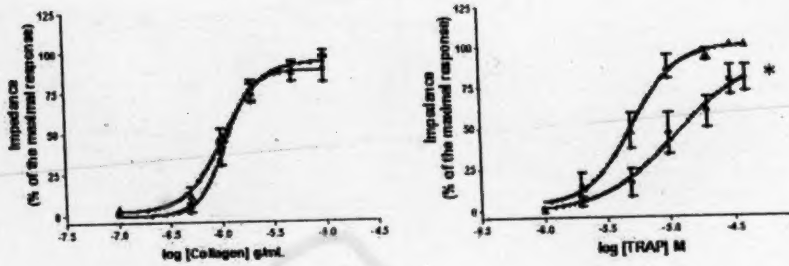
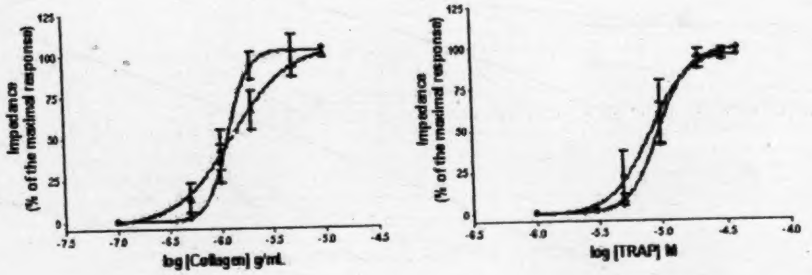


Figure 47. Effects of NO and PGI_2 on platelet aggregation in whole blood induced by collagen and TRAP. A: control; B: NO; C: PGI_2; D: NO + PGI_2. Data are mean ± SEM, n = 5-18. (Triangle: prior CPB samples; Circle: post CPB samples). * $p<0.05$ post CPB versus prior CPB.

C. PGI$_2$ treatment



D. NO + PGI$_2$ treatment



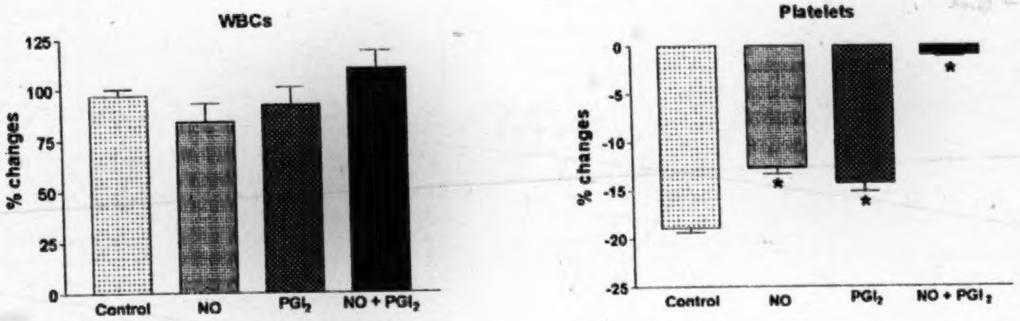
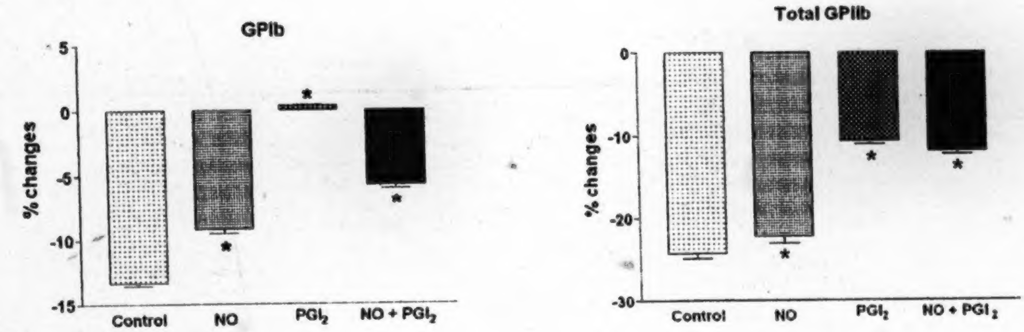


Figure 48. Effects of NO and PGI_2 on leukocyte and platelet numbers during CPB. Effects of the treatment are calculated as the percent changes between the prior CPB and the post CPB samples (shown on Figure 45). Data are mean ± SEM, n = 5-18. * $p<0.05$ treatments versus control.



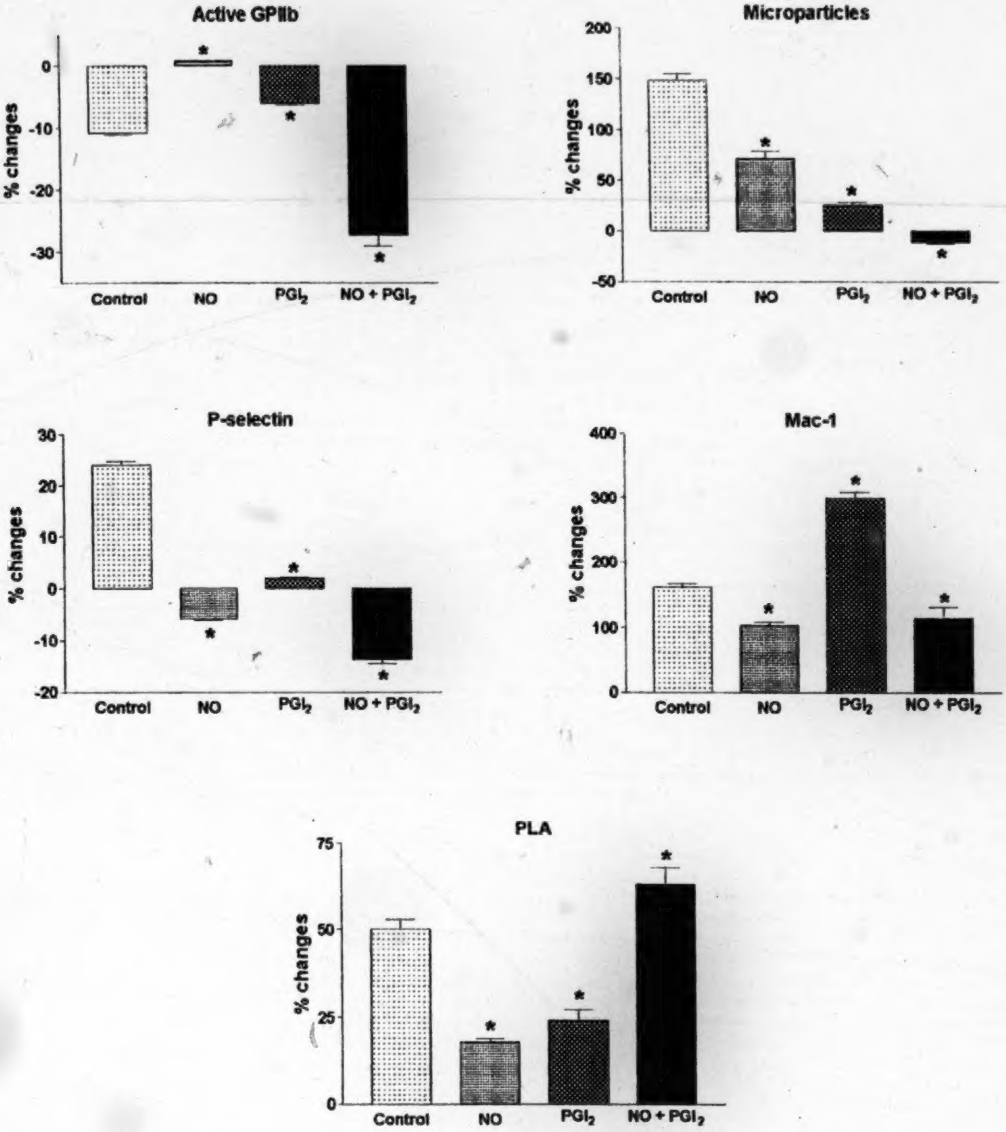


Figure 49. Effects of NO and PGI_2 on the expression of major platelet receptors (GPIb, total GPIIb, active GPIIb, P-selectin), microparticle formation, leukocyte Mac-1, and PLA during CPB. Effects of treatments are calculated as the percent changes between the prior CPB and the post CPB values (shown on Figure 46). Data are mean ± SEM, n = 5-18. * $p<0.05$ treatments versus control.

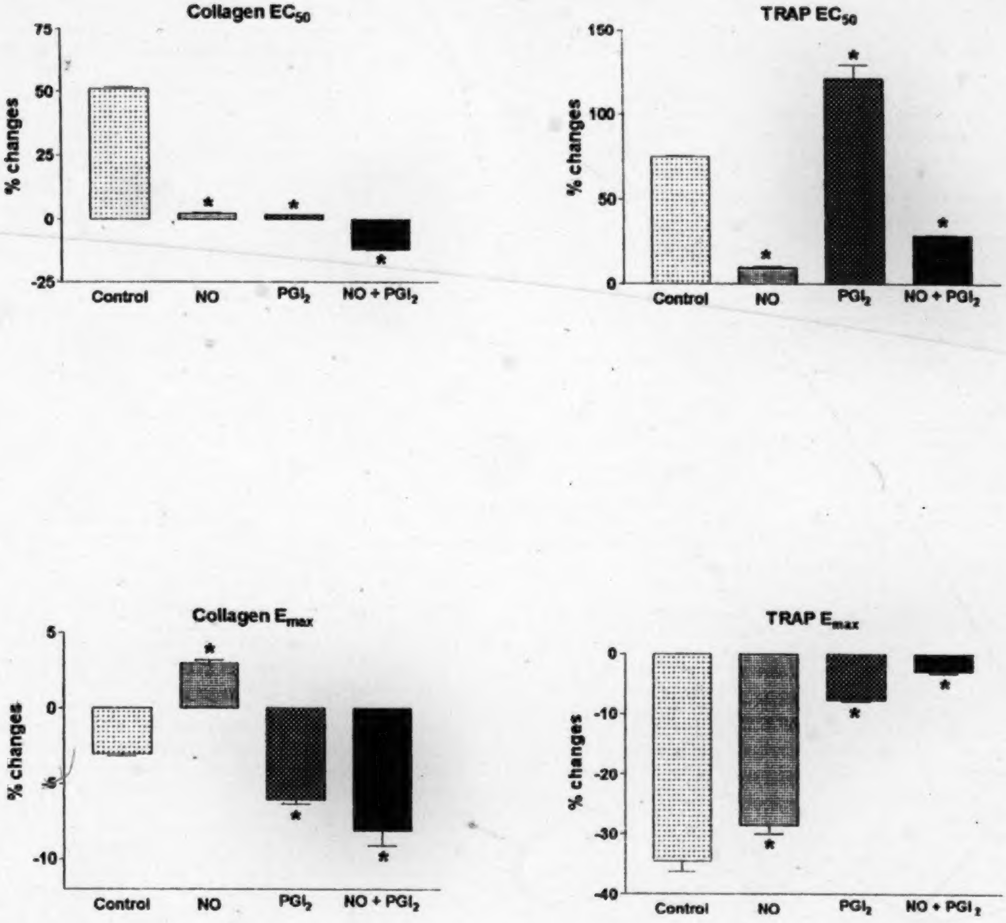


Figure 50. Effects of NO and PGI_2 on platelet aggregation in whole blood induced by collagen and TRAP. Effects of treatments are calculated as the percent changes of EC_{50} and E_{max} between the prior CPB and the post CPB values (shown on Table 6). Data are mean ± SEM, n = 5-18. * $p<0.05$ versus control.

Table 7. Effects of NO and PGI_2 on cAMP and cGMP levels in cell-free plasma and blood cell samples during CPB.

Cell-free plasma

	Prior CPB		Post CPB	
Treatment	cAMP	cGMP	cAMP	cGMP
Control	95 ± 7	87 ± 9	100 ± 3	96 ± 11
NO	98 ± 5	92 ± 8	104 ± 5	100 ± 5
PGI_2	103 ± 6	99 ± 3	96 ± 5	99 ± 5
NO + PGI_2	99 ± 2	96 ± 2	98 ± 2	95 ± 8

Blood cell

	Prior CPB		Post CPB	
Treatment	cAMP	cGMP	cAMP	cGMP
Control	86 ± 8	90 ± 10	92 ± 5	92 ± 5
NO	91 ± 6	93 ± 7	104 ± 11	100 ± 3
PGI_2	94 ± 5	100 ± 4	100 ± 9	100 ± 5
NO + PGI_2	90 ± 3	91 ± 3	94 ± 6	90 ± 3

Effects of Heparin

Since heparin has been shown to influence platelet aggregation (Khuri *et al*, 1995; Upchurch *et al*, 1997) we studied platelet and leukocyte function prior and after administration of heparin. Since all subjects enrolled into our study received heparin prior the administration of NO and iloprost, the data analyzed in this chapter were pooled into two groups: Pre Heparin and Post Heparin group.

We showed that the administration of heparin resulted in a significant increase in WBC numbers (5%), a reduction in platelet count (6%), an upregulation of total (20%) and active GPIIb/IIIa (12%) and an increase in Mac-1 (7%) (Table 8). Moreover, for TRAP-induced platelet aggregation, the EC_{50} values were reduced by approximately 3-fold and E_{max} was increased by 41%. The similar effects could not be observed in collagen-induced aggregation (Table 9 and Figure 51).

Table 8. Effects of heparin on platelet and leukocyte functions

	Sample	
Parameters	Pre Heparin	Post Heparin
WBCs	6.49 ± 0.40	6.83 ± 0.43 #
Platelets	221 ± 9	207 ± 8 #
GPIb	818 ± 25	826 ± 28
Total GPIIb	508 ± 17	611 ± 26 #
Active GPIIb	4.22 ± 0.19	4.73 ± 0.23 #
MP	4.41 ± 0.51	5.11 ± 0.60 #
P-selectin	13.9 ± 0.6	15.2 ± 0.8 #
Mac-1	292 ± 14	312 ± 19 #
PLA	0.325 ± 0.033	0.284 ± 0.030 #

Data are mean ± SEM, n = 25-41. Number of WBCs and platelets are expressed as 10*9/L. GPIb, total GPIIb, active GPIIb, P-selectin and Mac-1 level are expressed as MFI; MPs are reported as the percent of PE-positive cells in the gated region of the 10000 events. Formation of PLA is expressed as 10*9/L. # $p<0.05$ post heparin versus pre heparin samples.

Table 9. Effects of heparin on collagen- and TRAP-induced platelet aggregation in whole blood: EC_{50} and E_{max} values.

		Sample	
Treatment		Pre Heparin	Post Heparin
Collagen	EC_{50}	1.14 ± 0.02	1.14 ± 0.03
	E_{max}	95.0 ± 3	95.2 ± 2
TRAP	EC_{50}	21.8 ± 7.1	6.91 ± 0.42 #
	E_{max}	75.2 ± 5.2	102 ± 10 #

Collagen EC_{50} is expressed as µg/mL, while TRAP EC_{50} is in µM. E_{max} values are calculated as a percent of the maximal responses in the patient. Data are mean ± SEM, n = 25-41. # $p<0.05$ post heparin versus pre heparin samples.

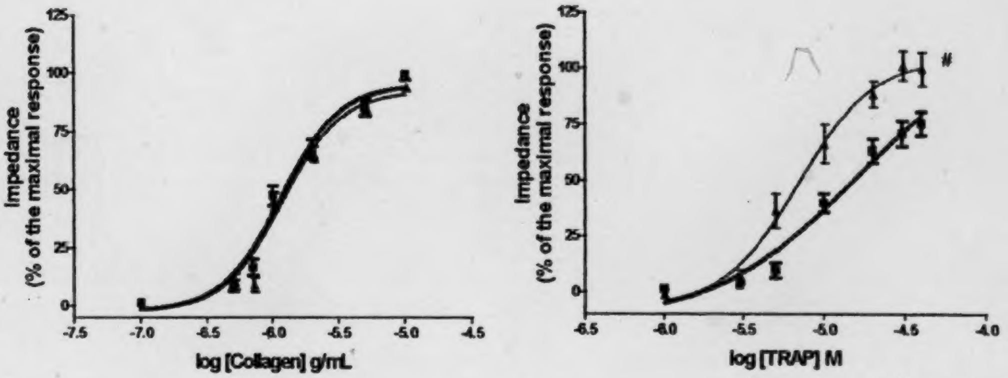


Figure 51. Effects of heparin on collagen- and TRAP-induced platelet aggregation in whole blood: concentration-response curves. Data are mean ± SEM, n = 25-41. (Square: pre heparin samples; Triangle: post heparin samples). # $p<0.05$ post heparin versus pre heparin samples (repeated two-way ANOVA).

Discussion

Mechanisms of PAR agonist actions on human platelets

Stimulation of platelet function by PAR agonists

In the first part of this study, we investigated the mechanisms of action of PAR agonists including thrombin, TRAP, PAR1AP and PAR4AP on human platelets. These agonists induced platelet aggregation in a concentration-dependent manner. To our knowledge, this is the first report showing a comparison of concentration-response curves for PAR1 and PAR4 agonists in human platelets. The complex nature of interactions between thrombin, PAR peptides, their receptors and the down-stream cellular signalling pathways presented challenges to this classical pharmacological approach. In addition, PAR agonists such as SFLLRN can be readily cleaved and inactivated by plasma- and serum- associated aminopeptidase leading to decreased aggregating activity. Indeed, the treatment of platelets with amastatin, an aminopeptidase inhibitor, has been shown to enhance SFLLRN-induced aggregation in platelet-rich plasma (Coller *et al*, 1993). However, in washed human platelets aminopeptidase did not affect the pharmacological activity of the PAR-APs, as shown by the lack of amastatin effect on PAR-AP-induced aggregation. These results allowed us to construct concentration-response curves to PAR peptides over a broad range of agonist concentrations and analyze the corresponding EC_{50} values.

Although all PAR agonists were equally effective in inducing aggregation, they differed in their aggregating potency. The comparison of EC_{50}s showed that thrombin was the most potent agonist followed by PAR1AP, TRAP and PAR4AP. The high potency of thrombin can be explained by the presence of the hirudin-like binding domain in the NH_2-terminal of PAR1 (Figure 8). Hirudin, a leech-derived anticoagulant, binds to thrombin's fibrinogen exosite thereby inhibiting fibrin clot formation. The hirudin-like domain of PAR1 is essential for the high affinity binding and the potent effects of thrombin on PAR1-containing cells (Vu *et al*, 1991b). Peptides not susceptible to thrombin cleavage but comprising this region, such as thrombomodulin and fibrinogen, block the actions of thrombin or thrombin-stimulated cleavage of receptors (Bouton *et al*, 1995). This hirudin domain is important in reducing the kinetic barrier to thrombin/receptor complex formation (Jacques *et al*, 2000), suggesting that thrombin binding at this site initiates a conformational change in the active center of the enzyme that accommodates the proteolytic cleavage sequence and facilitates binding (Ishii *et al*, 1995). In addition to this unusually high affinity-binding site, thrombin exerts a non-proteolytic interaction with GPIb complex (McNicol *et al*, 1989), which consists of the high affinity site located at the NH_2-terminal of the GPIbα subunit for thrombin interaction (Andrews *et al*, 1999). Although the interest in the role of the GPIb/IX/V complex in thrombin-induced platelet aggregation waned considerably after the discovery of proteinase-activated receptors, PARs alone cannot account for all of the thrombin actions on platelets. For example, at thrombin concentrations in the nanomolar range, thrombin-mediated responses absolutely require GPIb/IX/V, and the most convincing evidence for this requirement comes from the studies on platelets of patients suffering from Bernard-Soulier syndrome, a disease associated with alterations of GPIb/IX/V receptors (De Marco *et al*, 1991). Greco and colleagues (1996) have recently developed immunological reagents capable of selectively blocking thrombin binding to either PAR1 or GPIbα receptors and have found that thrombin binding to GPIbα is necessary for the rapid calcium mobilization observed in platelets at nanomolar concentrations of this aggregating agent. Thrombin binding to GPIb is also essential for the thrombin-induced procoagulant responses of platelets (Dormann *et al*, 2000).

Furthermore, the potency of thrombin is largely enhanced by its ability to act via a 'dual' PAR1/PAR4 receptor system in human platelets (Kahn *et al*, 1998; 1999). Thrombin-mediated proteolysis of PAR1 and PAR4 generates tethered ligands autostimulating both receptors. Indeed, the PAR1 NH_2-terminal peptide released by thrombin proteolysis can also activate platelets (Furman *et al*, 1998; 2000). Thus, thrombin-induced proteolysis of PAR1 and PAR4 can generate both the tethered ligands and the cleaved PAR1 peptide, which may potentiate each other and amplify thrombin-mediated aggregation (Furman *et al*, 1998).

Although PAR1AP, PAR4AP and TRAP can fully activate human platelets, their absolute potencies are relatively low, with EC_{50}s in the micromolar range. It is generally accepted that these comparatively low potencies are due to differences between a built-in tethered ligand and a ligand free in solution, which would gain an extra thermodynamic degree of freedom and be able to diffuse away from the receptor. Moreover, one cannot exclude alternative mechanisms of receptor activation, for example, receptor cleavage may release a 'constraint' that triggers the intracellular signal transduction cascade (Hollenberg, 2000). This suggestion is supported by the work of Lau *et al* (1994) who found that TRAP on its own, in contrast to thrombin, is not a full agonist for platelet activation and signal transduction in the absence of secretion of secondary mediators. We have found that PAR4AP is less potent than PAR1AP in inducing platelet aggregation. This is most likely due to the lack of hirudin-like domain in PAR4 (Figure 8). Indeed, PAR4 requires higher thrombin concentrations for activation than the other receptors (Kahn *et al*, 1998; Xu *et al*, 1998). The difference in potency may be related to coupling of the two receptors to their target G-proteins. PAR1 can couple to $G_{12/13}$, G_q and G_i (Hung *et al*, 1992), whereas PAR4, in the same cellular environment, cannot couple to G_i (Faruqi *et al*, 2000) (Figure 52A).

We also demonstrated that concomitant administration of subthreshold concentrations of PAR1AP and PAR4AP caused maximal aggregation. It has been shown that thrombin has a differential affinity for PAR1 and PAR4, such that activation of the latter receptor takes place only at comparatively high concentrations of thrombin arguing against a major role of PAR4 in thrombin signalling (Kahn *et al*, 1999). However, the potentiating interactions between PAR1AP and PAR4AP observed in our study further underscore the importance of PAR4 in the aggregation of human platelets by thrombin, even at concentrations that only minimally activate PAR4. Interestingly, mouse platelets that do not express PAR1 rely on the collaborative interactions between PAR3 and PAR4 during thrombin-induced aggregation (Nakanishi-Matsui *et al*, 2000). Moreover, the PAR4-deficient mouse shows a prolonged bleeding time, which is consistent with a key role of PAR4 in mouse haemostasis and thrombosis (Sambrano *et al*, 2001). Thus, activation of platelets by thrombin involves the interactions between PAR4 and either PAR1 (human) or PAR3 (mouse) to amplify aggregation.

Interestingly, there are some differences between PAR1 and PAR4 signaling. First, thrombin-mediated biphasic Ca^{2+} signaling can be resolved into a rapid PAR1-mediated signal and a slower sustained PAR4-mediated response, which serves to extend high intracellular Ca^{2+} levels and support the late phase of platelet aggregation (Covic *et al*, 2000). Second, signal generated by PAR4 is more persistent than that of PAR1 probably because the activation-dependent internalization of PAR4 is slower than that of PAR1. This is evidenced by the finding that PAR4 is less rapidly phosphorylated and desensitized following thrombin stimulation relative to PAR1 (Shapiro *et al*, 2000). Third, PAR4, but not PAR1, mediates platelet responses to leukocyte-derived cathepsin G, implicating PAR4 in platelet-leukocyte interactions at sites of vascular injury or inflammation (Sambrano *et al*, 2000). Therefore, the differences in the desensitization/internalization kinetics of PAR1 and PAR4 and the capability of PAR4 responding to other proteinases such as trypsin (Xu *et al*, 1998) suggest that PAR4 plays an important role in platelet function. Although PAR1 seems to be the predominant receptor involved in both platelet aggregation and coagulation, PAR4 may help to sustain aggregation or other platelet responses during the period when the local concentration of thrombin is high and PAR1 is rapidly desensitized.

Thrombin-induced aggregation is dependent upon glycoprotein receptors such as GPIb and GPIIb/IIIa. Therefore, we measured the expression of GP receptors on platelets stimulated with PAR agonists by flow cytometry. GPIb is a positive effector of PAR1-driven platelet activation by localizing thrombin to sites that facilitate cleavage of the nearby PARs (Gralnick *et al*, 1994). Thrombin down-regulates the surface expression of GPIb (De Marco *et al*, 1994; Michelson & Barnard, 1987). Our results showed a similar down-regulation of GPIb receptors after stimulation with thrombin and PAR-APs. The peptides caused a 40-50% decrease in GPIb surface expression, in association with platelet aggregation. The mechanism(s) of this down-regulation induced by PAR-APs remains to be studied, but this may be similar to that seen with thrombin, which is known to cause a rapid redistribution of GPIb complex into the surface-connected canalicular system (Hourdille *et al*, 1990).

Thrombin up-regulates and activates the GPIIb/IIIa complex (Suzuki *et al*, 1992). We demonstrated that PAR-APs also increased the surface expression of non-activated GPIIb/IIIa by 1.5-fold, and caused a dramatic up-regulation of activated GPIIb/IIIa by 20- to 80-fold. These results emphasize the importance of activation of GPIIb/IIIa receptors in mediating aggregation induced by PAR agonists. Similar to other ligands such as collagen and von Willebrand factor (Radomski *et al*, 2001), the changes in the ratio of GPIb to GPIIb/IIIa could be important in shifting platelet reactivity away from GPIb-mediated adhesion to GPIIb/IIIa-mediated aggregation.

Pharmacological regulation of PAR agonist-induced platelet aggregation

Prostacyclin and NO are the two major platelet inhibitors generated from endothelium (Jurasz *et al*, 2000). We used PGI_2 and NO-releasing agent, GSNO (Radomski *et al*, 1992), to study their effects on PAR-mediated aggregation. We found that although these inhibitors could effectively suppress aggregation, their effects on GPIIb/IIIa activation were variable. Indeed, inhibition of aggregation by PGI_2 correlated well with the reduction of IIb/IIIa activation in thrombin-, TRAP-, PAR1AP- and PAR1AP + PAR4AP-induced aggregation, but not with PAR4AP-induced aggregation. Moreover, GSNO caused only down-regulation of GPIIb/IIIa activation in platelets stimulated with TRAP, PAR1AP and PAR1AP + PAR4AP. PAR4-mediated upregulation of GPIIb/IIIa was insensitive to PGI_2 and NO, indicating that their inhibitory mechanisms affect other stages of platelet activation. Prostacyclin and NO stimulate the activity of adenylyl cyclase and guanylyl cyclase and this stimulation results in an increase in intracellular cAMP and cGMP levels, respectively (Best *et al*, 1977; Gerzer *et al*, 1988). The downstream signaling include cAMP-dependent protein kinases (PKA), cGMP-dependent protein kinases (PKG), cGMP-gated channels, cAMP-gated channels and perhaps still unknown effector systems (Radomski & Moncada, 1993; Armstrong, 1996). Human platelets contain very high concentrations of PKA and PKG, and selective stimulation of either PKA or PKG correlates with the inhibition of platelet activation and aggregation. In addition to inhibition of GPIIb/IIIa activation and fibrinogen binding, the mechanism of PKA- and PKG-mediated platelet regulation involves decreased actin polymerization and myosin light chain kinase activity. Furthermore, there is a reduction of intracellular Ca^{2+} via inhibition of intracellular and plasmalemmal calcium channels and activation of intracellular and plasmalemmal calcium pumps, as well as the interruption in the PLC/G-protein/receptor coupling (Colman *et al*, 1999; Jurasz *et al*, 2000).

We also found that aggregation induced by PAR-APs is significantly more sensitive to the inhibitory effects of PGI_2, as compared with those induced by thrombin and collagen. These differences were not detectable for inhibition of platelet aggregation by GSNO. The reasons for this differential reactivity are not clear.

We used ASA, phenanthroline and apyrase to explore the relative contribution of TXA_2, MMP-2 and ADP release to PAR agonist-induced aggregation. We observed that platelet aggregation stimulated by thrombin, TRAP, PAR1AP and PAR4AP was ASA-insensitive, indicating that these reactions were TXA_2-independent. In contrast to ASA, apyrase and phenanthroline partially inhibited aggregation induced by thrombin, TRAP and PAR1AP indicating that MMP-2 and ADP are mediators of these aggregatory reactions. However, even the three inhibitors were not capable to inhibit completely thrombin-, TRAP- or PAR1-induced aggregatory responses, suggesting the involvement of TXA_2-, ADP- and MMP-2-independent mechanism(s). Interestingly, PAR4AP-induced aggregation was (1) abolished in the presence of apyrase, an agent that degrades ADP (Whigham *et al*, 1976) and (2) was insensitive to phenanthroline. Moreover, there was a strong correlation between aggregation and ADP release during PAR4AP-induced aggregation. These data suggest that PAR4AP-mediated platelet aggregation is entirely ADP-dependent and metalloproteinase-independent.

ADP, when released from dense granules of activated platelets, acts through at least three receptors, ionotropic purinoceptors $2X_1$ ($P2X_1$), G_q-coupled purinoceptors $2Y_1$ ($P2Y_1$) and G_i-coupled purinoceptors $2Y_{AC}$ ($P2Y_{AC}$) (Kunapuli *et al*, 1998; Cattaneo & Gachet, 1999). Recent studies demonstrated that coactivation of both G_q and G_i (that inhibits the activity of adenylate cyclase, thus promoting aggregation) is a prerequisite for GPIIb/IIIa activation and ADP-mediated platelet aggregation (Jin *et al*, 1998; Pulcinelli *et al*, 1999). This is consistent with the strong aggregatory effects of thrombin, whose receptor couples to both G_i and G_q. In contrast, PAR4-mediated signal transduction pathway only involves G_q (Faruqi *et al*, 2000). We used ticlopidine, an ADP receptor antagonist known to inhibit aggregation (Defreyn *et al*, 1989) and epinephrine, which aggregates platelets via stimulation of α_2-adrenergic receptor and G_i in platelets (Steen *et al*, 1993), to probe further the mechanisms of PAR4-induced aggregation. We found that the blockade of purinergic receptors with ticlopidine abolished PAR4-induced aggregation and that this inhibition could be surmounted by the pre-treatment of platelets with epinephrine. These results implicate the ADP-G_i pathway in PAR4-induced aggregation of human platelets. Thus, it is likely that PAR4-mediated platelet aggregation is dependent upon the direct interactions of thrombin and PAR4APs with G_q and the indirect (ADP-mediated) stimulation of G_i (Figure 52).

Our results also demonstrated that PAR agonist-induced aggregation was ASA-insensitive. Despite its effectiveness in treating and preventing the thrombotic complications of atherosclerotic disease, a minority of patients appears to be relatively ASA-resistant even when it is administered in large doses (Helgason *et al*, 1993). Platelet aggregation studies have demonstrated the incomplete inhibition of aggregation in 25 % of patients prior ischemic stroke and these subjects were receiving long-term ASA therapy (Helgason *et al*, 1994). The mechanism(s) of the ASA-resistance is not clear, but probably is due to the TXA_2-independent activators of platelet aggregation (i.e. thrombin) bypassing the ASA-inhibitory effect and resulting in thrombosis (Radomski *et al*, 2000). We also showed that, although PAR agonist-induced aggregation is ASA-insensitive, a combination of this compound with ADP and MMP-2 inhibitors reduced aggregation. These observations further underscore the effectiveness of an anti-platelet approach that is based on the combinations of inhibitors of aggregation that act via different molecular mechanisms. Indeed, the results of a recent CURE study that included the combination of ticlopidine and aspirin to treat patients with stroke clearly emphasize the value of such therapy for the treatment and/or prevention of vascular disorders associated with platelet activation (Alberts & Easton, 2002).

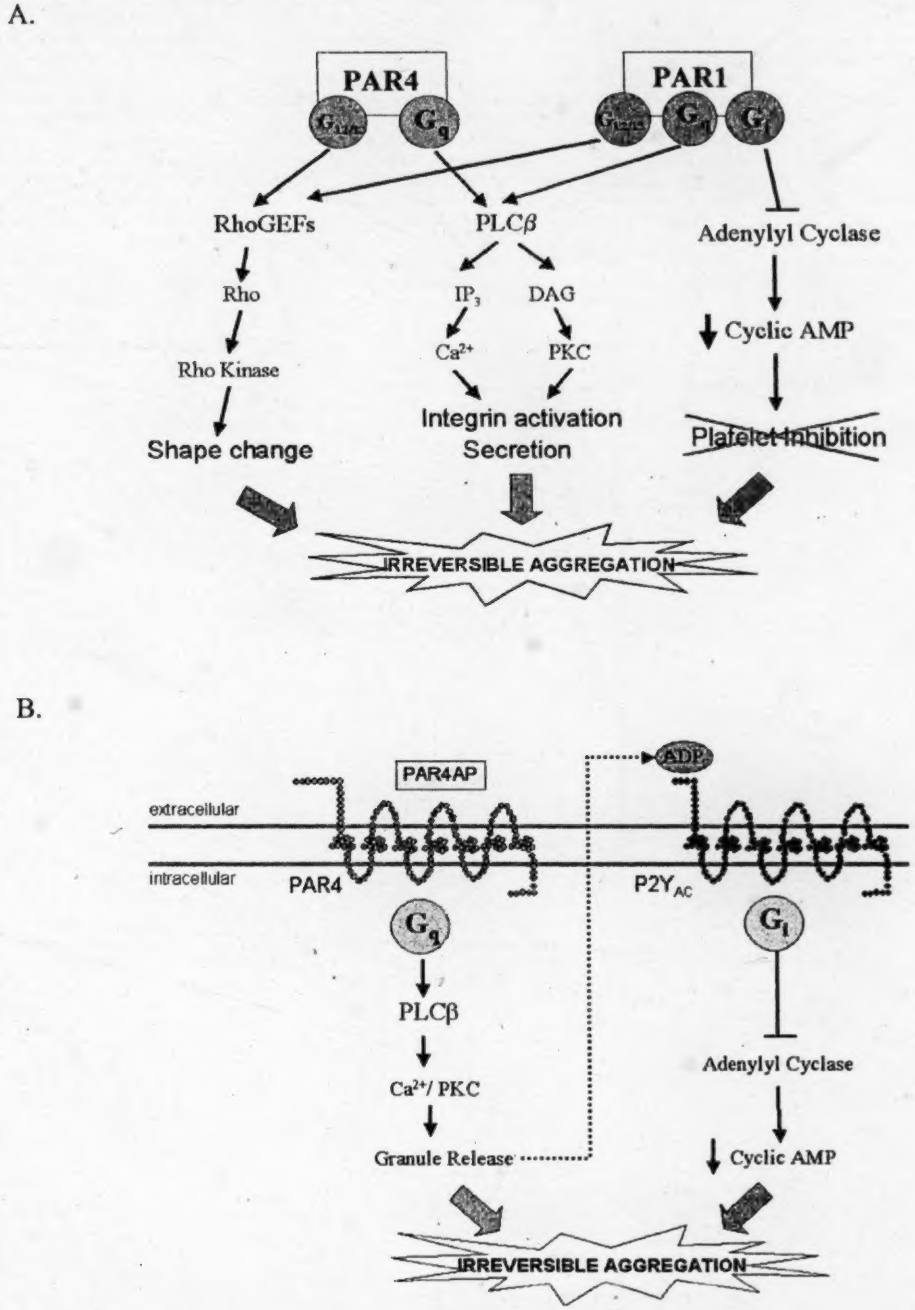

A.
PAR4
$G_{12/13}$
G_q
PAR1
$G_{12/13}$
G_q
G_i
RhoGEFs
Rho
Rho Kinase
Shape change
PLCβ
IP_3
DAG
Ca^{2+}
PKC
Integrin activation
Secretion
Adenylyl Cyclase
Cyclic AMP
~~Platelet Inhibition~~
IRREVERSIBLE AGGREGATION
B.
PAR4AP
ADP
extracellular
intracellular
PAR4
$P2Y_{AC}$
G_q
PLCβ
Ca^{2+}/ PKC
Granule Release
G_i
Adenylyl Cyclase
Cyclic AMP
IRREVERSIBLE AGGREGATION

Figure 52. Thrombin receptor signaling. A) Signal transduction pathways mediated by PAR1 and PAR4 (Coughlin, 2000; Faruqi *et al*, 2000). B) A hypothetical explanation of ADP-dependency in PAR4-mediated irreversible platelet aggregation.

PARs and MMPs in platelet-leukocyte interactions

Leukocytes modulate platelet function

In the vasculature, one of the main proteinase reservoirs is leukocyte. Leukocytes represent one of the first lines of defense against microbial invasion and infections. They contain a number of serine proteinases and MMPs that play important roles in leukocyte function. In quiescent cells, these proteinases are stored in the intracellular granules. Gelatinase-containing granules have been identified in polymorphonuclear leukocytes (Okada *et al*, 1997; Opdenakker *et al*, 2001). During inflammatory extravasation leukocytes secrete proteinases to affect their microenvironment. The secreted proteinases facilitate a number of leukocyte functions such as extravasation, migration, phagocytosis and killing of infectious agents (Kubes *et al*, 1993; Okada *et al*, 1997). A number of tissues or plasma polypeptides with strong and specific antiproteinase activities (Chignard *et al*, 1994; Owen & Campbell, 1995) normally ensure that neutrophil proteinases will only be active in the pericellular space, and will not diffuse to the surrounding tissues or vessels (Owen *et al*, 1995). The leukocyte-derived proteinases are the major modulators of platelet responses (Chignard *et al*, 1986), and the participation of leukocytes in the thrombotic and vaso-occlusive diseases has received more attention in recent years (Nash, 1994). Leukocytes have been proposed as one of the cellular components involved in the initiation and propagation of deep venous thrombosis (Stewart, 1993). During extracorporeal circulation, both platelets and neutrophils can be activated and form circulating mixed aggregates, which can be responsible for the plugging of microvessels (Gawaz *et al*, 1994). Increasing evidence also indicates that leukocytes play an important role in the deposition and aggregation of platelets on an injured arterial wall, and predisposing the ischemia-reperfusion injury (Merhi *et al*, 1994; Carden & Granger, 2000).

For these reasons, we studied the mechanisms involved in regulation of platelet-leukocyte interactions. Two experimental approaches were used to study these interactions. First, we used light aggregometry to determine if leukocytes exerted effects on PAR agonist-induced platelet aggregation. In these experiments, isolated leukocytes were added to PAR agonist-stimulated washed platelets. The addition of physiological amounts of leukocytes (i.e. platelet: leukocyte ratio between 60:1 and 100:1), led to potentiation of PAR agonist-induced platelet aggregation. The release of platelet activating factor, a known platelet agonist (Montrucchio *et al*, 2000) could account for this effect of leukocytes.

In contrast to physiological numbers, higher amounts of leukocytes that simulated inflammation-induced leukocytosis (platelet:leukocyte ratios 30:1 and 5:1) inhibited platelet aggregation. The addition of ODQ, a selective inhibitor of soluble guanylyl cyclase (Moro *et al*, 1994), partially reversed this inhibition suggesting the involvement of NO (Radomski *et al*, 1996). The secretion of leukocyte proteinases such as cathepsin G and elastase could also account for the inhibitory effects of leukocytes (Del Maschio *et al*, 1990). Indeed, elastase and cathepsin G remove the von Willebrand factor binding site from GPIb, and delete the tethered ligand domain from thrombin receptors (i.e. PAR1) (Molino *et al*, 1993; 1995), thus rendering the receptors less responsive to thrombin.

We also employed flow cytometry to determine PAR agonist-induced formation of heterotypic aggregates (monocyte-platelet and neutrophil-platelet) by a combination of platelet-specific and leukocyte-specific MoAbs. Unlike the optical aggregometry that does not differentiate between platelet-platelet and platelet-leukocyte aggregates, flow cytometry allows one to investigate the dynamics of both platelet-platelet and platelet-leukocyte interactions (Ault *et al*, 2001). In addition, flow cytometry can detect the 'preaggregatory' reaction, which is defined as the formation of the first small aggregates consisting of very few platelets and leukocytes while light aggregometry lacks the sensitivity to detect the formation of small aggregates (Gear, 1981; Matzdorff *et al*, 1998). It is worth to mention that in this study we used the MoAb against a pan-leukocyte specific receptor, CD45, to identify the total leukocyte population without differentiation between mononuclear and polymorphonuclear cells. This experimental approach allowed us to investigate the presence of all types of leukocytes interacting with platelets; however, we could not differentiate between specific subpopulations of leukocytes involved in these heterotypic interactions. This is important since monocytes and neutrophils form heterotypic aggregates with platelets more readily than lymphocytes (Rinder *et al*, 1991). Moreover, in a vascular injury model, there is a specific accumulation of circulating monocytes and polymorphonuclear leukocytes on platelet thrombi (Kirchhofer *et al*, 1997).

Platelets modulate leukocyte function

As shown by our results, leukocyte activation is also biphasically regulated by platelets (Figure 24A). A number of researchers have shown that platelets may also regulate leukocyte activation. Leukocytes that have formed aggregates with platelets produced higher hydrogen peroxide levels than did non-aggregate-forming neutrophils (Nagata *et al*, 1993). Platelets potentiate leukocyte recruitment in the mesenteric circulation during ischemia-reperfusion (Salter *et al*, 2001). Moreover, it has been demonstrated that platelet-derived growth factor, platelet factor 4, TXA_2 and serotonin are involved in neutrophil accumulation, degranulation, phagocytosis and respiratory burst (Mannaioni *et al*, 1997). On the other hand, platelet-derived NO, transforming growth factor-β and PDGF may contribute to inhibition of neutrophil function by platelets.

Role of P-selectin and microparticles in platelet-leukocyte interactions

Close heterotypic cell-cell associations such as platelet-leukocyte interactions require the presence of specific receptors that serve as molecular bridges between the two cell types. Platelets play an active role in this process by expressing P-selectin and releasing membrane-shedding MPs, which are markers of platelet activation and found to be associated with platelet dependent thrombotic disorders (Chong *et al*, 1994). P-selectin, a component of the α granule membrane of resting platelets, is rapidly translocated to the platelet membrane surface during platelet activation. P-selectin mediates the initial tethering between two cells and triggers leukocyte activation via interactions with specific carbohydrate ligands on leukocyte surface (PSGL-1) (Sako *et al*, 1993). *In vivo*, P-selectin has been shown to be important in neutrophil recruitment to the sites of platelet deposition (Konstantopoulos *et al*, 1998). Moreover, P-selectin expression promotes shear-induced platelet aggregation (Merten *et al*, 2000a), increases the stability of platelet aggregates and initiates thrombus growth (Merten *et al*, 2000b). Furthermore, the engagement of P-selectin on activated platelets and PSGL-1 on monocytes generates outside-in signals to induce the expression of tissue factor (Celi *et al*, 1994; Lindmark *et al*, 2000) and cytokines (Weyrish *et al*, 1996). Finally, studies using animal models showed that pharmacological inhibition of P-selectin function attenuated myocardial ischemia-reperfusion injury (McKenzie & Gurbel, 2001). Thus, P-selectin and its ligand PSGL-1 play an important role in the leukocyte recruitment in vascular disorders.

We found that PAR agonist-induced stimulation resulted in a significant (10-12 fold) up-regulation of P-selectin expression on the platelet surface. This increase was at least 4-times higher when compared to collagen-induced P-selectin expression. The reasons for this differential effectiveness (PAR agonists v/s collagen) are not clear. These results further evidence the importance of PAR agonists as major mediators of the cellular interactions between platelets and leukocytes in hemostasis and thrombosis.

Upon stimulation, platelet plasma membrane is shed to generate MPs. Although substantially smaller than the intact platelets, MPs carry platelet specific glycoprotein receptors that are important in promoting platelet-platelet, platelet-leukocyte and platelet-vessel wall interactions (Merten *et al*, 1999; Forlow *et al*, 2000). MPs generated by thrombin-activated platelets stimulate calcium translocation, and inositol phosphate formation in target cells (Barry *et al*, 1999). Monocyte chemotaxis and leukocyte-leukocyte interactions also depend on platelet MPs, which are considered major carriers of platelet-activating factor that activates leukocytes (Iwamoto *et al*, 1996; Montrucchio *et al*, 2000). Barry *et al* (1997) showed that arachidonic acid in MPs induced cyclooxygenase-2 and prostacyclin production in endothelial cells and stimulated monocyte adherence through upregulation of adhesion molecule (ICAM-1) (1998). Interestingly, MPs play an important role in fibrin formation and accelerate thrombin generation (Sims *et al*, 1988; Gilbert *et al*, 1991), since they provide a stable procoagulant surface (Nieuwland *et al*, 1997). Therefore, platelet MPs participate in the multicellular interactions involving platelets, leukocytes and the endothelium. We studied the capacity of PAR agonists to induce MP formation by platelets and found that TRAP-induced MP formation was significantly lower than that of other PAR agonists. It is widely accepted that TRAP may not mimic all aspects of thrombin-induced platelet activation, because the tethered ligand receptor may not be the only platelet receptor for thrombin (Lau *et al*, 1994). However in contrast to TRAP, stimulation of platelets with even more selective PAR1AP and PAR4AP resulted in the release of MPs in amounts comparable to that of thrombin. Therefore, the difference (TRAP versus other PAR agonists) in the efficacy to generate MPs may lie down-stream from PARs. Indeed, TRAP stimulates a transient activation of the platelet Na^+/H^+ exchange that is accompanied by a transient increase in intracellular calcium, and these effects of TRAP are reversible (Lau *et al*, 1994). In contrast, the effects of thrombin on these signaling pathways are irreversible and long-lasting (Nieuwland *et al*, 1994). In addition, Gemmell *et al* (1993) has shown that MP formation involves fibrinogen binding to GPIIb/IIIa receptors. We have found that TRAP-induced upregulation of GPIIb/IIIa receptors is significantly lower than those of thrombin, PAR1AP and PAR4AP (Figure 19).

Prostacyclin and NO as inhibitors of PAR-induced platelet-leukocyte interactions

We used PGI_2 and NO to study the susceptibility of PAR agonist-induced platelet-leukocyte aggregation to inhibition with these agents. We found that these inhibitors reduced the aggregation. They also abolished MP formation. Inhibition of PLA and MP formation by PGI_2 and NO did not correlate well with the reduction of P-selectin expression induced by each PAR agonists. Indeed, PAR4AP-mediated P-selectin expression was GSNO insensitive. Moreover, neither GSNO nor PGI_2 could reduce P-selectin expression completely. These data suggest that NO and PGI_2 may also inhibit PLA formation in a P-selectin independent manner and raise the possibility that other regulatory mechanisms, which are sensitive to the inhibition of PGI_2 and GSNO, may contribute to PLA. Although selectins help the initial margination and rolling of leukocytes in the bloodstream (Dore *et al*, 1993), activated β_2-integrins are necessary for stable adhesion and spreading of the flowing leukocytes on the immobilized platelets (Kuijper *et al*, 1996, Krieglstein & Granger, 2001). Integrins are heterodimeric proteins consisting of noncovalently associated α and β subunits. Leukocytes constitutively express three integrins sharing a common β_2 subunit (CD18), but each with a unique α subunit: LFA-1 (Lymphocyte function-associated antigen-1, CD11a/CD18), Mac-1 (macrophage-antigen 1, CD11b/CD18) and glycoprotein 150,95 (CD11c/CD18). The physiological significance and cellular ligands for Mac-1 are well known, while those for LFA-1 and glycoprotein 150,95 are not fully characterized (Gahmberg *et al*, 1998). Leukocyte β_2-integrins play an important role in platelet-activating factor-induced hypotension, leukopenia, hemoconcentration, and intestinal necrosis, and Mac-1 is the main adhesion molecule involved in the pathogenesis of this injury (Sun *et al*, 1996). Monoclonal antibodies against Mac-1 can inhibit the adhesion-strengthening interactions between neutrophils and surface-immobilized platelets by more than 90% (Diacovo *et al*, 1996). Recent clinical investigations have implicated the upregulation of Mac-1 on leukocytes of the patients with acute myocardial infarction and post-coronary angioplasty in the pathogenesis of these disease states (Simpson *et al*, 1988; Lefer *et al*, 1993; Neumann *et al*, 1995). Moreover, pharmacological modulation of PLA with the

GPIIb/IIIa inhibitor abciximab, NO and PGI_2, all inhibited Mac-1 upregulation and improved microvascular reflow, thus providing myocardial salvage in animal models of acute myocardial infarction (Simpson *et al*, 1988; Lefer *et al*, 1993). Taking into consideration all these data, in conjunction with our studies using NO and PGI_2, we propose that PAR agonist-induced PLA formation follows the classical principles of platelet-leukocyte interactions. First, the primary adhesion of platelets to leukocytes is mediated by tethering of platelet's P-selectin to PSGL-1 on leukocytes. This heterotypic interaction is likely further stabilized by binding Mac-1 to counter-receptor(s) (i.e. fibrinogen-GPIIb/IIIa, GPIbα, ICAM-2 and junctional adhesion molecule-3) on platelets (Weber & Springer, 1997; Kuijper *et al*, 1998; Simon *et al*, 2000; Santoso *et al*, 2002). Prostacyclin and NO synergize as inhibitors of platelet aggregation both *in vitro* (Radomski *et al*, 1987b) and *in vivo* (Spiecker *et al*, 1993). We found that these inhibitors also exerted synergistic effects as inhibitors of platelet-leukocyte aggregation and P-selectin translocation. Some clinical studies support a close link between the levels of NO and PGI_2 and P-selectin expression in platelets. For example, the reduced NO levels, a risk factor for silent cerebral infarction in patients with atrial fibrillation, are linked to P-selectin expression on resting platelets (Minamino *et al*, 1998). Moreover, continuous PGI_2 therapy can prevent increased plasma P-selectin levels in pulmonary arterial hypertension (Sakamaki *et al*, 2000). The remarkable ability of NO and PGI_2 to down-regulate platelet activation and PLA formation further provides rationale for combined antithrombotic therapies using these compounds or their analogues.

Role of MMPs, TXA_2 and ADP-dependent pathways in PAR-induced platelet-leukocyte interactions

To determine the relative contribution of TXA_2-, MMPs and ADP-dependent pathways to PAR-mediated platelet-leukocyte aggregation, we used the respective inhibitors of these pathways such as aspirin (cyclooxygenase inhibitor), phenanthroline (MMP inhibitor) and apyrase (ADP scavenger). The inhibitors differentially reduced this aggregation, with phenanthroline being the most potent. Therefore, we investigated the role of MMPs in platelet-leukocyte interactions. Since leukocytes are known to express a number of MMPs, (Fujisawa *et al*, 1999; Pei, 1999; Opdenakker *et al*, 2001) we have focused on MMP-1, 2, 3 and -9 as potential mediators of PLA formation. Studies using Western immunoblot showed that both latent and active forms of these MMPs are expressed in leukocytes. Stimulation of PLA formation with PAR agonists led to the liberation of active, but not latent, MMPs to the releasate of PLA. The flow cytometry data provided evidence for the expression of MMPs on the surface of PLA. A very short time course (10 min duration for the aggregation experiments) of the appearance of active MMPs in the PLA milieu suggests that the release of these proteinases from cellular stores, but not *de novo* synthesis, is responsible for this phenomenon. Most MMPs are synthesized and released into the extracellular space as a proenzyme or zymogen form. These pro-MMPs are bound to specific (tissue inhibitors of metalloproteinases) or non-specific (α_2-macroglobulin) inhibitors within the extracellular space and remain latent until activated. The activation process of MMPs is an important regulatory step, requiring a number of integrated and step-wise biochemical events to occur in order to yield a fully active MMP. Therefore, we suggest that PAR agonists induce the translocation of pro-MMPs (either from platelets or leukocytes) to the surface of PLA where these proteinases are activated.

There is, however, some evidence for the *de novo* MMP synthesis during platelet-leukocyte interactions. Galt *et al* (2001) demonstrated that activated platelets deliver discrete signals to monocytes leading to the synthesis of MMP-9. In addition, activated platelets express a α-granule-associated CD40L (Henn *et al*, 1998), which upon engagement with CD40 present on leukocytes induces MMPs activation. The discovery of the translocation of CD40L not only provides an alternative pathway for MMPs to mediate platelet-leukocyte interactions, but also points to a possible role of this protein in the pathogenesis of acute coronary syndromes (Aukrust *et al*, 1999). The rupture of an atherosclerotic plaque with subsequent thrombosis is an important pathogenic event in the development of acute coronary syndromes (Ross, 1999). The MMPs family probably plays a crucial role in determining the integrity of the tissues in the plaque (Davies, 1998). The CD40L has been shown to be associated with the activation of MMPs in macrophages and vascular smooth muscle cells, but this protein does not seem to affect the expression of TIMPs (Mach *et al*, 1997). Therefore, the platelet-leukocyte interactions involving the CD40-CD40L engagement may lead to MMP activation, which ultimately promotes plaque rupture and the development of acute coronary syndromes. Once released by PAR stimulation, leukocyte MMPs appear to act as mediators of platelet-leukocyte interactions. The inhibitory effects of neutralizing MoAbs against MMP-1, 2 and 3 provide further argument in support of the proaggregatory roles of these MMPs. This is consistent with the known stimulator roles of MMP-1 and MMP-2 in platelet adhesion and aggregation (Sawicki *et al*, 1997, 1998; Radomski *et al*, 2002;

Jurasz *et al*, 2002; Kazes *et al*, 2000; Galt *et al*, 2002). In contrast to MMP-1 and MMP-2, MMP-9 acts as an inhibitor of platelet aggregation (Fernandez-Patron *et al*, 1999a). Our experiments using low concentrations of MoAbs against MMP-9 suggest that this proteinase may also inhibit platelet-leukocyte aggregation. However, in the presence of higher concentrations of anti-MMP-9 antibodies PAR-induced aggregation appears to be inhibited suggesting that MMP-9 could exert a biphasic (stimulator/inhibitor) effect on platelet-leukocyte aggregation. The pharmacological experiments exploring the effects of MoAbs against MMP-1, MMP-2 and MMP-3 on PLA showed that each of the Abs on its own resulted in a strong (60-75%) inhibition of aggregation. The experiments with phenanthroline that inhibits all these MMPs also suggest that the maximal level of inhibition of platelet-leukocyte aggregation is approximately 75%. It is unlikely that MoAbs used in these experiments exerted non-specific effects on the aggregation since this possibility was ruled out in control experiments using IgG_1. Moreover, we have no evidence for reciprocal cross-reactivity of antiMMP-1, 2 and -3 antibodies. Therefore, it is possible to suggest that the simultaneous release of these three MMPs is necessary for platelet-leukocyte interaction.

To probe further the effects of MMPs on platelet-leukocyte aggregation we used purified human MMP-1, -2, -3 and -9. As with platelet aggregation (Sawicki *et al*, 1997; Kazes *et al*, 2000; Radomski *et al*, 2002; Galt *et al*, 2002), incubation of purified MMPs with platelet-leukocyte suspensions did not result in aggregation. However, MMP-2 and MMP-3 greatly potentiated the effects of subthreshold concentrations of PAR agonists on the aggregation. These effects are again similar to the actions of MMP-2 on platelets (Sawicki *et al*, 1997; Kazes *et al*, 2000). The requirement of PAR agonists to unravel the effects of MMPs in platelet-leukocyte suspensions indicates that a cellular target for the action of these enzymes is expressed upon cell activation. Interestingly, only MMP-2 and MMP-3 amplified the pro-aggregatory effects of PAR agonists. In contrast, MMP-1 and MMP-9 did not influence the extent of aggregation. This differential effect of MMPs is likely to be reagent-dependent. The commercially available preparations of MMP-2 and MMP-3 are supplied as a mixture of zymogen and active enzyme, while MMP-1 and MMP-9 are prepared as zymogen mainly. Activation of these proenzymes can be performed using trypsin or p-aminophenylmercuric acetate (APMA); however, since both of these reagents affect platelet function and activate other leukocyte-derived proteinases (Ogata *et al*, 1995; Duncan *et al*, 1998; Sawicki *et al*, 1997), this activating procedure could not be used in our experiments.

The mechanisms of MMP action on PLA remains to be elucidated. Since active, but not latent, enzymes exert their effects on PLA, it is likely that these actions are related to the proteolytic properties of MMPs. MMPs hydrolyze Gly-Leu and Gly-Ile bonds, thus degrading the extracellular matrix proteins such as collagen that contain such domains (Lauer-Fields *et al*, 2000). In addition to extracellular matrix, MMPs can also cleave other proteins such as big endothelin-1 (Fernandez-Patron *et al*, 1999b), calcitonin-gene-related peptide (Fernandez-Patron *et al*, 2000) and monocyte chemoattractant protein-3 (McQuibban *et al*, 2000). The cleavage results in protein activation (big-endothelin-1) or degradation (calcitonin-gene related peptide and chemoattractant protein-3). The interactions of MMPs with cell surface receptors have also been described. The association of MMP-1 with α2β3 integrin, a receptor for collagen, confines this proteinase to the points of cell contact with collagen, so that the ternary complex of integrin, proteinase, and substrate function together to regulate extracellular matrix degradation and migration (Stricker *et al*, 2001; Conant *et al*, 2002). Strongin's group showed that MT1-MMP via interactions with $\alpha(v)\beta3$ integrin promotes MMP-2 maturation (Deryugina *et al*, 2001), and enhances tyrosine phosphorylation of focal adhesion kinase (Deryugina *et al*, 2002). Furthermore, MT1-MMP serves as integrin convertase, which induces cleavage and maturation of integrin $\alpha(v)$ subunit (Deryugina *et al*, 2002; Ratnikov *et al*, 2002). Our group has shown that platelet integrin receptors GPIb and GPIIb/IIIa (Radomski *et al*, 2001, Martinez-Cuesta *et al*, 2001) are likely to be activated by MMP-2. In addition, MMP-1 is known to stimulate tyrosine phosphorylation, thus promoting clustering of β_1-integrins to focal adhesion points (Galt *et al*, 2002). All these actions may contribute to PLA-promoting effects of MMPs.

Summing up, our results indicate that MMPs play an important role in PLA. The proposed sites of MMPs action on PLA are depicted in Figure 53. These interactions are likely to be important not only for vascular homeostasis, but also in such diverse pathologies including thrombosis, atherosclerosis, carcinogenesis and inflammation.

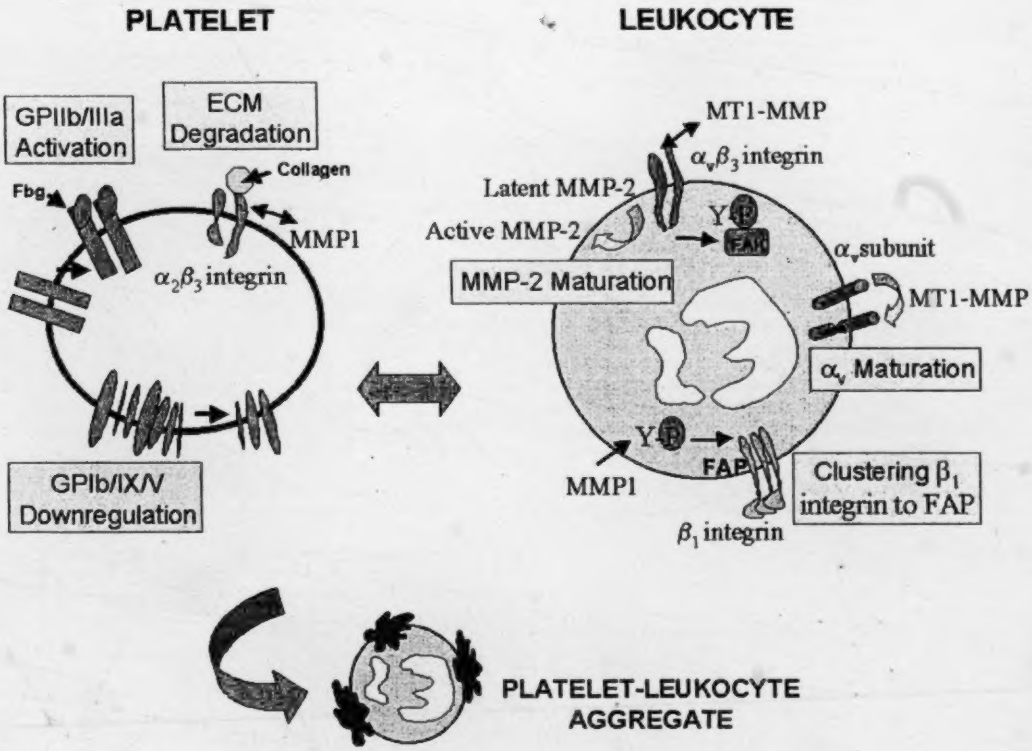

PLATELET
LEUKOCYTE
GPIIb/IIIa Activation
ECM Degradation
Collagen
Fbg
MMP1
$\alpha_2\beta_3$ integrin
GPIb/IX/V Downregulation
MT1-MMP
$\alpha_v\beta_3$ integrin
Latent MMP-2
Active MMP-2
Y-P
FAK
MMP-2 Maturation
α_v subunit
MT1-MMP
α_v Maturation
Y-P
FAP
MMP1
Clustering β_1 integrin to FAP
β_1 integrin
PLATELET-LEUKOCYTE AGGREGATE

Figure 53. Potential sites for MMP action on the formation of PLA (FAP: focal adhesion point; FAK: focal adhesion kinase; Y-P: tyrosine-phosphorylation, Fbg: fibrinogen; ECM: extracellular matrix).

Pathogenesis of thrombotic and inflammatory lesions caused by cardiopulmonary bypass surgery

Platelet and leukocyte functions during CPB

Advances in extracorporeal life support have made cardiopulmonary bypass (CPB) one of the most common procedures in cardiac surgery worldwide. The procedure; however, carries a certain risk inherent to its use. Indeed, CPB is a form of controlled inflammatory and thrombotic injury brought about by the contact of blood with foreign surface of the extracorporeal circuit. The CPB represents a unique type of injury whose precise timing is controlled. Clinically, cognitive decline as a complication of CPB has been a serious concern since the advent of this procedure (Selnes *et al*, 1999). Among other serious complications both post-operative thrombosis and hemorrhage have been reported (Woodman & Harker, 1990; Despotis *et al*, 1996b). Potentially, mechanisms responsible for these complications of CPB may involve the intersecting pathways of coagulation, fibrinolysis, complement and inflammation. In our investigation, we examined markers of platelet and leukocyte functions to get a better insight into the pathogenesis of cellular lesion of CPB. Prompted by a very high effectiveness of the combination of NO and PGI_2 in attenuating platelet and leukocyte activation *in vitro*, we have conducted a pilot clinical study to evaluate the effects of these inhibitors on platelet and leukocyte function prior and following CPB in patients with the coronary artery disease.

We found that the surgery led to a significant reduction in platelet count, accompanied by a dramatic increase in WBC numbers. Thrombocytopenia is well documented in association with CPB. Early hemodilution occurs from the use of crystalloid fluid for priming the extracorporeal circuit. However, Holloway and colleagues (1988) suggested that this decrease in platelet count could not be fully explained by hemodilution; mechanical disruption, as well as adhesion to the extracorporeal circuit along with platelet sequestration in organs contribute to the true drop in circulating platelet counts (Hennessy *et al*, 1977). The reduction of platelet counts is also common in cases with extensive tissue damage (i.e. extensive operative trauma, multiple fractures, and severe burns), in which the formation of platelet aggregates can lead to a consumption of platelets (Addonizio & Colman, 1982; Utley, 1990). The same pathogenic mechanism may take place with the implantation of large vascular grafts that provide vast foreign surfaces capable of activating platelets. It is important to know that a marked decrease in the platelet count is a regular phenomenon after major trauma and major surgery, particularly if the surgery involves multiple blood transfusions or insertion of porous vascular grafts (Despotis *et al*, 1996a and b).

The increase in WBC numbers is associated with the inflammatory response triggered by the surgery (Despotis *et al*, 1996b). The contact of circulating immune cells with foreign surface of CPB circuit first leads to localized inflammatory response characterized by the release of complement fragments and cytokines such as interleukin-6 and TNF-α (Tonnesen *et al*, 1996; Vinten-Johansen & Buckberg, 2000). These inflammatory mediators, in turn, mobilize systemic inflammatory reactions, in which leukocytes are recruited and activated, generate superoxide radicals and other cytokines, and adhere to the vascular endothelium. The acute influx of neutrophils into lung tissue and the activation of the complement cascade have been evidenced and may contribute to the lung injury following the CABG surgery (Ratliff *et al*, 1973). Interestingly, there is also evidence for specific myocardial dysfunction brought about by CPB, and the release of inflammatory mediators such as NO and MMP may contribute to the pathogenesis of this heart lesion (Mayers *et al*, 1999, 2001). The adhesion of leukocytes to the endothelium is mediated by specific adhesion molecules, Mac-1, expressed on the leukocyte surface (McEver, 2001), and the CABG surgery is known to result in increased Mac-1 levels (Gillinov *et al*, 1993). In addition, the use of heparin and protamine may also contribute to blood cell damage, thus impairing hemostasis. The degree of platelet activation and thrombocytopenia increases in a dose-dependent manner with increasing concentrations of heparin (Chong & Ismail, 1989). Infusion of protamine sulfate for neutralization of heparin induces thrombocytopenia (Mendeloff *et al*, 1994). Heparin-protamine complexes also activate the complement pathway, which causes leukocyte activation (Cavarocchi, 1985; Cook *et al*, 1992).

Cardiopulmonary bypass also impairs platelet function. In this study, platelet function was tested using collagen and TRAP as aggregating agents. Since thrombin-induced aggregation results in fibrin formation in whole blood and this interfere with the impedance assay of platelet aggregation. TRAP, which does not directly stimulate the coagulation system was used instead of thrombin. The choice of collagen and a PAR agonist to probe platelet function during CPB was not accidental. First, both collagen and thrombin are highly concentrated at the sites of vascular injury. Second, collagen, one of the major subendothelial matrix components, is exposed in the damaged vessels and available for platelet adhesion and aggregation. It is important to mention that the collagen used in this clinical study was native collagen fibrils (type I) from equine tendons, not from human. And finally, thrombin is one of the major proteinases generated during CPB (Boisclair *et al*, 1993). The major stimulus for thrombin generation is the activation of the extrinsic coagulation pathway both from contact of blood with synthetic surface of CPB apparatus and from exposure of blood to the surgical wound (Edmunds *et al*, 1982). The analysis of whole blood platelet aggregation in response to collagen and TRAP showed that the responses were depressed after the surgery, as evidenced by increased EC_{50} values, decreased E_{max} values and a left shift of the concentration-response curves. Decreased platelet function has been reported with respect to both aggregation and adhesion (Kabakibi *et al*, 1998; Uthoff *et al*, 1994). In fact, both functional (platelet reactivity) and quantitative (platelet count) defects in the primary hemostasis are considered to be the major mechanisms for postoperative bleeding in patients undergoing CPB (Kabakibi *et al*, 1998; Ray *et al*, 1994). The underlying mechanisms of these abnormalities of platelet function are not clear, but can be associated with prolonged platelet activation leading to 'platelet exhaustion' during the surgery (Weerasinghe & Taylor, 1998). Ferraris and colleagues (1998) have suggested that CPB-induced bleeding is related to the desensitization of thrombin receptors, since thrombin generation is seen during the surgery. Therefore, prolonged platelet activation induces receptor desensitization and renders platelets less responsive to agonists. Using flow cytometry, we analyzed the molecular pattern of CPB-induced platelet injury. We found that CPB markedly enhanced platelet receptor activation and degranulation. This was evidenced by increased expression of surface P-selectin, MP formation and internalization of GPIb. In addition, we detected decreased expression of GPIIb/IIIa complex that appears to be related to the reduced aggregatory responses to both collagen and TRAP. At the same time we found that CPB led to leukocyte activation as evidenced by increased WBC numbers and Mac-1 levels. In keeping with simultaneous platelet and leukocyte activation, we measured increased PLA formation. This may lead to the plugging of capillaries, reduction of local blood flow and cellular necrosis. Moreover, PLA formation further supports the generation of tissue factor and triggers the coagulation cascade (Rosing *et al*, 1985; Chang *et al*, 1993). All these data clearly point to crucial contributions of platelet and leukocyte dysfunctions in the CPB lesion.

Effects of NO and iloprost

In the second interventional arm of our clinical study, we investigated whether the pharmacological treatment of CPB patients with potent endothelial regulators, NO and PGI_2, could attenuate CPB-induced platelet and leukocyte dysfunctions. There is compelling evidence that the endothelium is critical to normal vascular wall homeostasis. Endothelial cells synthesize and release a number of factors, including PGI_2, NO, bradykinin, endothelium-derived hyperpolarizing factor (EDHF), TXA_2 and endothelin, which are important in the regulation of vascular tone and control of platelet and leukocyte adhesion, aggregation and migration (Jurasz *et al*, 2000). Impaired endothelial function leads to enhanced thrombogenicity due to increased adhesion of activated platelets and leukocytes. In addition, impaired bioavailability of endothelium-derived NO and activation of the endothelin system results in an enhanced vasoconstrictor tone and impaired regulation of organ blood flow (Fernandez-Patron *et al*, 1999a). In the treatment groups, we used NO and iloprost, a stable analogue of PGI_2. The rationale for the choice of these mediators is based on the fact that patients undergoing cardiac surgery often have underlying diseases such as hyperlipidemia, hypertension, and atherosclerosis. Endothelial dysfunction plays a major role in the pathological profile of these medical conditions, and both NO and PGI_2 deficiencies have been implicated in the pathogenesis of these vascular disorders (Egashira, 2002). We decided to use NO gas and iloprost, administered directly to the blood phase of oxygenator, as pharmacological tools to inhibit CPB-induced blood cell activation. Inhaled NO is now routinely used in the intensive medicine in order to combat increased pulmonary vascular resistance that is a hallmark of a number of adult and neonatal pathologies (Sahebjami, 1993; Modi & Dore, 1999; Jiang *et al*, 2002). Furthermore, there is evidence that inhaled NO can attenuate the development of the coronary thrombosis (Adrie *et al*, 1996). Finally, a number of investigators have shown that the addition of NO gas into oxygenator sweep phase will exert platelet-sparing effects and protect patients undergoing extracorporeal circulation procedures from thrombocytopenia (see below).

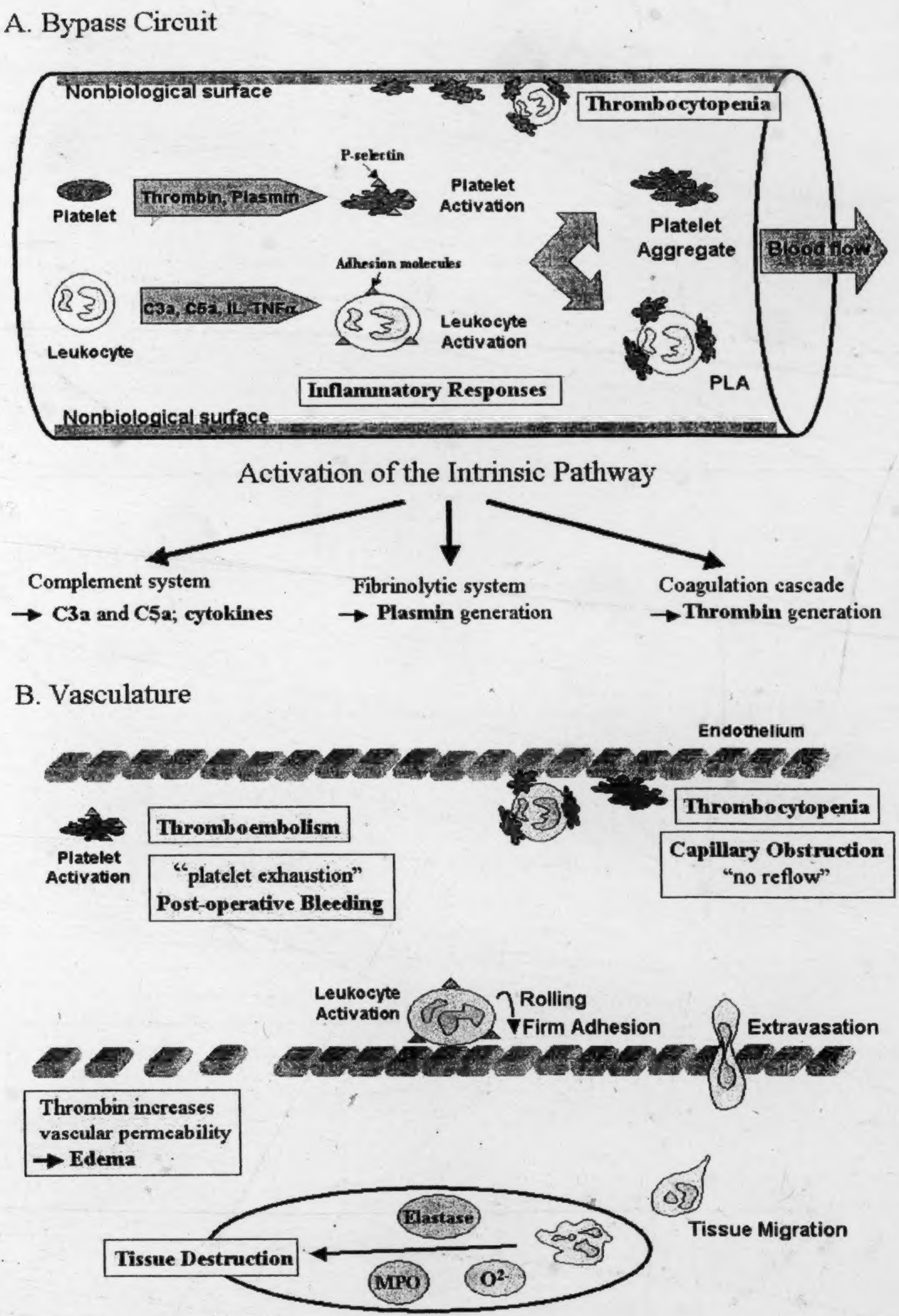
Preparations of intravenous prostacyclin have been successfully used in extracorporeal circuits to reduce platelet activation (Bernabei *et al*, 1995). The major problems confounding the systemic use of prostacyclin have been its potent hypotensive effect and a short (min) biological half-life (Armstrong, 1996). Iloprost is a stable prostacyclin analogue that has a longer half-life (Schror *et al*, 1981). Because of its potential vascular effects, we decided to use low (2 ng/kg/min) amounts of this drug. The concept of simultaneous use of NO and PGI_2 has stemmed from our earlier (Radomski *et al*, 1987) and current (presented in this thesis) pharmacological data that clearly showed that the combined use of both agents represents the most efficacious strategy to protect platelets from activation.

We found that NO gas and iloprost when used individually did not exert a direct effect on leukocyte function. Indeed, neither agent was capable of attenuating CPB-induced leukocytosis or increase in Mac-1 levels. In contrast, these compounds directly attenuated CPB-induced platelet dysfunction. Nitric oxide and iloprost decreased the degree of thrombocytopenia and preserved collagen-mediated aggregatory responses. This was accompanied by inhibition of CPB-induced GPIIb/IIIa and GPIb changes and reduction of MP formation and P-selectin expression. In keeping with the reduction of P-selectin translocation, PLA levels were reduced by NO or iloprost. The beneficial effects of NO and PGI_2 have also been demonstrated in other clinical studies. For instance, Aren and colleagues (1983) have shown that an infusion into the bypass circuit of PGI_2 dramatically reduces plasma level of β-thromboglobulin, reflecting less platelet activation, although the dosage used in this study (50ng/Kg/min) was much higher than the one we used (2ng/Kg/min). In a recent study, iloprost has been combined with a GPIIb/IIIa receptor antagonist at a clinically safe dose, and shown to inhibit platelet activation during *in vitro* extracorporeal circulating (Bernabei *et al*, 1995). Using NO gas, Mellgren and colleagues (1996) demonstrated higher platelet counts in experimental perfusion circuits treated sweep gas containing NO (15-75 ppm); however, platelet function was not measured in their study. Inhaled NO has been shown to downregulate P-selectin expression, platelet aggregation and fibrinogen binding in patients suffering from severe adult respiratory distress syndrome (Gries *et al*, 1998). It should be noted that the concentrations of NO (100-884 ppm) being used there was much higher than those we used (20 ppm). These platelet-sparing effects may not be detectable under all clinical conditions and could be accompanied by some vascular side-effects. For example, infusion of NO releasing agent, GSNO, into the CPB system did not prevent platelet activation (Langford *et al*, 1997). Moreover, DiSesa (1984) showed that PGI_2 infusion during CPB induced hypotension. These problems could be due to the dosage, the length of drug administration, the assessments of platelet functions, and the modes of drug administration (Mellgren *et al*, 1998). Summing up, the analysis of our results, in conjunction with the literature, shows that low amounts of NO gas and iloprost can be useful to attenuate CPB-induced platelet injury. Moreover, although NO and iloprost do not appear to influence leukocyte activation directly, both of them attenuate P-selectin-mediated PLA; thus, indirectly limiting the degree of leukocyte activation.

As mentioned before, NO and PGI_2 synergize with each other as inhibitors of platelet aggregation and inducers of disaggregation (Radomski *et al*, 1987b; Macdonald *et al*, 1988). Their cellular effects are mediated by the increase in intracellular cAMP and cGMP level, which can be hydrolyzed by phosphodiesterases (PDEs). In platelets, there are three types of PDEs with different affinities for cAMP and cGMP: cGMP-specific-PDE, cGMP-stimulated PDE (PDEII), and cGMP-inhibited PDE (PDEIII) (Colman *et al*, 1999). PDEIII is the most abundant; cGMP can utilize the cAMP pathway by inhibiting PDE in order to accumulate cellular cAMP level and enhance its biological actions. Therefore, the synergistic relationship between NO and PGI_2 could be mediated by cGMP-induced inhibition of type III PDE, an enzyme responsible for cAMP degradation in platelets (Maurice & Haslam, 1990; Spiecker *et al*, 1993). However, to our knowledge no data are available on the effects and mechanisms of actions of combination of NO and PGI_2 in the clinical setting.

Analysis of the combined NO-iloprost treatment on platelet and leukocyte functions has yielded both expected and unexpected results. As expected, based on the results of our *in vitro* experiments, the combined treatment was more effective than the single-drug treatment in further limiting the extent of thrombocytopenia, inhibiting active GPIIb/IIIa expression, formation of MP and reducing P-selectin expression. Since neither NO or iloprost affected leukocyte activation, it is not surprising that the combined treatment failed to exert the inhibitory effects on leukocytes. However, we found that the treatment increased PLA. It is difficult to rationalize this finding since the combined treatment inhibited P-selectin upregulation. Although the stabilization of PLA, following initial P-selectin-PSGL-1 interactions can take place via P-selectin-independent, Mac-1-dependent binding to platelet counterreceptors (Neumann *et al*, 1999). There is no data to suggest that NO and iloprost have the capacity to stimulate such a binding.

The data presented in this dissertation show that although the combined NO-iloprost treatment effectively inhibits CPB-induced platelet activation (and as such offers some advantages over the single drug treatment) it is not capable of attenuating leukocyte activation and its interactions with platelets. Therefore, additional strategies should be sought to block these reactions. One such approach has been suggested recently by Neumann and colleagues (1999) who showed that the blockade of GPIIb/IIIa receptor with abciximab results in cross-inhibition of Mac-1 binding to platelets. Other experimental treatments may involve the use of MMP inhibitors as evidenced in the second part of this thesis. Figure 54 depicts some of the platelet and leukocyte pathways that may lead to thrombotic and inflammatory injury of CPB and a possible pharmacological modulation by NO, prostacyclin, GPIIb/IIIa antagonists and MMP inhibitors of this injury.


A. Bypass Circuit
Nonbiological surface
Thrombocytopenia
P-selectin
Platelet
Thrombin, Plasmin
Platelet Activation
Platelet Aggregate
Blood flow
Adhesion molecules
Leukocyte
C3a, C5a, IL, TNFα
Leukocyte Activation
PLA
Inflammatory Responses
Nonbiological surface
Activation of the Intrinsic Pathway
Complement system
→ C3a and C5a; cytokines
Fibrinolytic system
→ Plasmin generation
Coagulation cascade
→ Thrombin generation
B. Vasculature
Endothelium
Platelet Activation
Thromboembolism
"platelet exhaustion"
Post-operative Bleeding
Thrombocytopenia
Capillary Obstruction
"no reflow"
Leukocyte Activation
Rolling
Firm Adhesion
Extravasation
Thrombin increases vascular permeability
→ Edema
Elastase
Tissue Migration
Tissue Destruction
MPO
O2-

C.

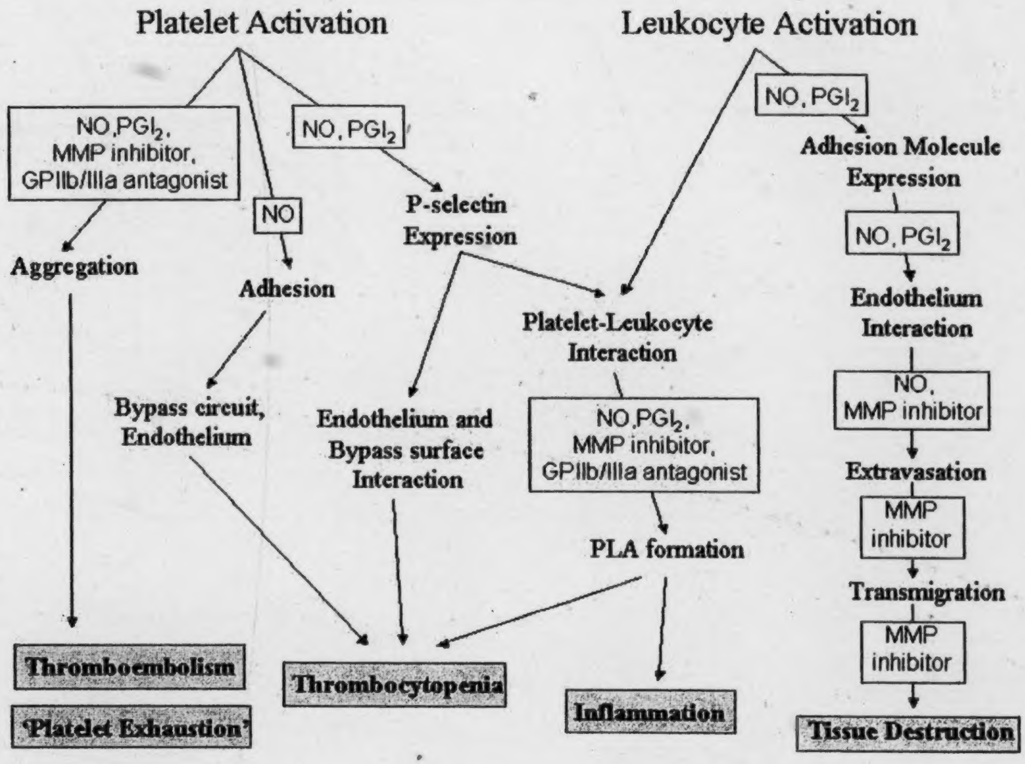

Platelet Activation
Leukocyte Activation
NO,PGI2, MMP inhibitor, GPIIb/IIIa antagonist
NO
NO, PGI2
NO, PGI2
Aggregation
Adhesion
P-selectin Expression
Adhesion Molecule Expression
NO, PGI2
Endothelium Interaction
Platelet-Leukocyte Interaction
Bypass circuit, Endothelium
Endothelium and Bypass surface Interaction
NO,PGI2, MMP inhibitor, GPIIb/IIIa antagonist
NO, MMP inhibitor
Extravasation
PLA formation
MMP inhibitor
Transmigration
MMP inhibitor
Thromboembolism
'Platelet Exhaustion'
Thrombocytopenia
Inflammation
Tissue Destruction

Figure 54. Potential mechanisms of CPB-induced thrombotic and inflammatory responses mediated by the activation of platelets and leukocytes (IL: interleukin; TNF: tumor necrosis factor; MPO: myeloperoxidase; O^{2-}: superoxide radical). A) Blood contact with the nonbiological surface of the extracorporeal system during CPB initiates the intrinsic pathway, which directly or indirectly activates three intersecting plasma proteinase pathways: Complement, fibrinolytic and coagulation system. Each generates active proinflammatory mediators through a series of consecutive proteolytic cleavages. Activation of platelets and leukocytes triggers the inflammatory responses, the formation of platelet aggregates and PLA. The adherence of activated platelets on the bypass circuit may also contribute to thrombocytopenia. B) Within the vasculature, activated platelets interact with other platelets or blood cells enhancing the thromboembolic state. Prolonged platelet activation results in 'platelet exhaustion', which relates to the post-operative bleeding as illustrated in my discussion. Similarly, the activated platelets and leukocytes together aggregate on the endothelium in the circulation, contributing to the 'no reflow' phenomenon and capillary obstruction. Thrombin generated from the coagulation cascade induces shape changes of endothelial cells, increased vascular permeability, resulting in diffuse and specific organ edema. On the other hand, activated leukocytes with the upregulation of surface adhesion molecules (i.e. Mac-1) attach to endothelium and undergo extravasation into the neighbourhood tissue. Release of superoxide radicals, myeloperoxidase, and elastase would all contribute to endothelial cell injury and tissue damage. C) Summary of the clinical effects of CPB-induced activation of platelets and leukocytes. The potential sites of actions of NO, PGI_2, GPIIb/IIIa antagonists and MMP inhibitors are highlighted.

In order to elucidate if the observed effects of NO and PGI_2 on platelets and leukocytes were cGMP/cAMP-dependent, we measured the concentrations of these cyclic nucleotides in both cell-free plasma sample and blood-cell sample using enzyme immunoassay. Interestingly, in all treatment groups, no significant increase in these nucleotides was observed. This may be explained, in part, by hemodilution during extracorporeal circulation, as well as by low dosages of NO and iloprost that were not sufficient to increase the levels of cyclic nucleotides. However, the possibility that some observed effects of NO and iloprost were cyclic nucleotide-independent could not be discounted by the present experiments. Finally, the absence of the changes of cyclic nucleotide levels may be due to the method of blood collection. We collected the blood sample from the radial arterial line, while both drugs were given directly to the oxygenator or into the blood phase close to the oxygenator. Although we added IBMX (3-Isobutyl-1-methylxanthine, a potent phosphodiesterase inhibitor) and EDTA (calcium chelating agent that inhibits phosphodiesterase activity) to the sample immediately after collection to suppress the activities of phosphodiesterases, cyclic nucleotides might have been already degraded by phosphodiesterases when blood circulated from the oxygenator to the radial artery.

Effects of heparin

We have also investigated the acute effects of heparin on platelet function during CPB. Current therapeutic interventions to reduce the incidence of hemorrhagic-thrombotic complications during CPB aim at inhibiting the activation of both the coagulation cascade and platelets. Systemic administration of heparin or the use of heparin-bonded circuits have been shown to blunt the complement cascade, neutrophil and platelet activation, fibrinolysis, and chemokines and cytokines expression in patients undergoing CABG. However, potential damaging effects of this anticoagulant on blood cells, especially platelets have been documented (Khuri *et al*, 1995). Some authors suggested that excessive bleeding complicating some CPB procedures was associated with the higher doses of bovine heparin; others found no differences in blood loss when either bovine or porcine heparin was used (Despotis *et al*, 2001). The clinical usefulness of heparin-coated CPB circuits is also uncertain. Van der Kamp and van Oeveren (1993) found no difference in platelet activation using this type of circuit, whereas Moen demonstrated platelet activation to be less pronounced (Moen *et al*, 1997).

Before I discuss our data on the effects of heparin on platelet and leukocyte function, some general pharmacological features of heparin need to be highlighted. Heparin, isolated from either porcine intestine or from bovine lung as an unfractionated array of different molecules, is the most commonly used anticoagulant to prevent clotting during cardiac or vascular surgery (Hirsh, 1991). Heparin is an acidic polysaccharide with side groups, either sulfates or N-acetyl groups, attached to individual sugar groups. The main advantage of heparin is that its action can be reversed immediately by protamine. Protamine, the only clinically available neutralizing agent, is a basic polypeptide isolated from salmon sperm (Levy, 1999). It is comprised mostly of arginine, protamine and is capable of reversing heparin action by a nonspecific acid-base interaction. Heparin acts indirectly as an anticoagulant by binding to antithrombin III (ATIII), thus enhancing the rate of thrombin-ATIII complex formation by 1000 to 10000 times (Weitz, 1994). Several other steps in the coagulation cascade, including clotting factor X are also inhibited albeit to a lesser degree by ATIII. Moreover, heparin binds to and activates components of the fibrinolytic system (Khuri *et al*, 1995). The resulting increased plasminogen activator function leads to the generation of plasmin, which binds to the surface of platelet causing α-granule release (Gouin *et al*, 1992). Heparin also binds to a number of blood cells and endothelial proteins including high molecular weight kininogen, vWf, plasminogen, fibronectin, lipoproteins, and platelet and endothelial receptors (Andersson *et al*, 1979). These drug-protein interactions are thought to be associated with an increased risk of bleeding after heparin administration, since the interaction of blood cells and the endothelium is crucial in maintaining vessel wall integrity and hemostasis. As a result, careful monitoring of a patient's anticoagulation status and adjustment of the heparin dosage during cardiac surgery is necessary. In addition, heparin exposure in susceptible patients may produce a rare but potentially serious complication, heparin-induced thrombocytopenia (HIT) (Chong, 1995). HIT is an adverse effect of heparin administration produced by antibodies (IgG) to the complex of heparin-platelet factor 4 leading to the formation of immune complexes. These immune complexes bind to platelets through platelet Fc receptors producing intravascular platelet activation, thromboemboli, and thrombocytopenia.

We have clearly shown that the administration of heparin resulted in thrombocytopenia and significant platelet activation, as evidenced by the upregulation of platelet activation markers and the potentiation of TRAP-induced platelet aggregation. That an acute decrease in platelet count occurs after heparin administration is due to its proaggregatory effect (Follis & Schmidt, 2000). The resulting platelet activation may lead to or predispose the phenomena of "platelet exhaustion". In fact, *in vitro*, heparin binds to platelets and, depending on the experimental conditions, can either induce or inhibit platelet aggregation (Hirsh *et al*, 2001). The results of our investigation not only support the notion that heparin administration during the use of extracorporeal circulation may cause platelet activation and further dysfunction, but also suggest agonist-dependent heparin effects on platelet responses. Therefore, the rationale of using heparin-coated circuit to attenuate platelet activation and preserve platelet function during CPB procedure appears questionable.

Study limitations

The major limitation of this study was the relatively small number of patients in the treatment groups. We were unable to correlate the results of laboratory tests with hard clinical outcomes such as bleeding and other thrombotic or inflammatory complications. However, our study has been designed as a pilot investigation to evaluate the clinical feasibility and pharmacological efficacy of combined treatment using low amounts of two powerful antiplatelet agents, NO and iloprost. Remarkably, even this experimental number was of sufficient statistical power to clearly delineate differences in platelet functions and platelet-leukocyte interactions between the control and the treatment groups.

Conclusions

We have studied the mechanims of action of PAR agonists including thrombin, TRAP, PAR1AP and PAR4AP on platelet aggregation and platelet-leukocyte interactions. Thrombin, TRAP and PAR1AP stimulation resulted in aggregation that was mediated via activation of thromboxane A_2, ADP and MMP-2 dependent pathways. In contrast, platelet aggregation induced by PAR4AP was ADP-dependent. PAR1AP and PAR4AP synergized to induce platelet aggregation. Thus, PAR activation induces aggregation that is differentially transduced in platelets. The interactions between PAR1 and PAR4 play a pivotal role in platelet-platelet interactions.

Stimulation of platelets and leukocytes with PAR agonists resulted in platelet-leukocyte aggregation; therefore, receptors of the PAR family act as a link between blood coagulation and inflammation. In contrast to platelet aggregation, platelet-leukocyte aggregation appears to be mostly mediated by the release of MMPs including MMP-1, MMP-2, MMP-3 and MMP-9. The high dependence of platelet-leukocyte interactions on MMPs indicates the roles of these enzymes in PLA under both physiological and pathological conditions.

In the latter part of this thesis, I have shown that the use of CPB in cardiac surgery is clearly associated with platelet and leukocyte activation. This activation may be decreased by pharmacological administration of NO and iloprost. The combined use of these compounds further attenuates the activation of platelet, but not leukocyte. The research conducted in this thesis shows that an increased understanding of platelet biology and pharmacology may assist in the management of various vascular disorders associated with platelet aggregation and platelet-leukocyte interaction.

Future Directions

I. Non-TXA_2, ADP, MMP-2 aggregatory pathways during PAR activation

TXA_2, MMP-2 and ADP are the major platelet aggregation mediators, and they are specifically inhibited by ASA, phenanthroline and apyrase. However, we found that aggregation induced by other PAR agonists (except PAR4AP) can be diminished by at most 50% even in the presence of the combination of ASA, phenanthroline and apyrase. This supports the existence of non-TXA_2, ADP, MMP-2 aggregatory pathways.

A proteinase-dependent pathway could be involved, since previous investigations have shown that platelet aggregation is potentiated by the proteinases released and activated during platelet activation.

As an example, both proelastase and elastase are present in the cytosol of intact platelets. During platelet activation, the elastase activity is upregulated due to the action of an unidentified trypsin-like proteinase (Legrand *et al*, 1977). Platelets are also known to contain serine proteinase inhibitors such as α_1-antitrypsin, and α_2-macroglobulin (Bagdasarian & Colman, 1978; Nachman & Harpel, 1976). Interestingly, elastase activity is suppressed by α_2-macroglobulin (James *et al*, 1986). Moreover, a Ca^{2+}-activated proteinase, calpain, is translocated to the plasma membrane and activated during thrombin stimulation. Croce *et al* (1999) suggested a regulatory role for calpain in early cytoskeletal remodelling, including secretion, shape change and aggregation.

Therefore, in addition to the MMP-2/-9 regulatory mechanism, I hypothesize that platelets possess other activating mechanisms involving the serine proteinase/antiproteinase system that may supplement the effects of MMPs.

II. Interactions of platelets, leukocytes and endothelium during thrombin stimulation: Highlighting the roles of thrombin in angiogenesis

We have shown that at least MMP-1, -2, -3 and –9 play important roles in modifying the PAR-mediated platelet-leukocyte interaction. Next, we would like to investigate if the endothelium is involved in platelet-leukocyte interaction. The rationale for this set of experiments is based on the fact that in the vascular system, endothelial cells are yet another reservoir of MMPs, which may influence the platelet-leukocyte interactions. Moreover, members of the PAR family are highly expressed in the endothelium and the interactions of thrombin with endothelial cells are central to its multifunctional role in the cardiovascular system. The role of MMPs in the interactions between platelet, leukocytes and endothelial cells and their regulation by thrombin may particularly be important in angiogenesis.

Angiogenesis, the formation of new blood vessels, is a complex process that occurs during physical and pathological situations. For instance, angiogenesis occurs during normal wound healing, embryonic development, vascularization of solid tumors, and collateral blood vessel formation. It has been proposed that proteinases are essential mediators of angiogenesis as they cause 1) local degradation of basement membrane allowing migration of endothelial cells out of the exiting vessel, 2) migration of endothelial cells through stroma, and 3) remodelling of the basement membrane as the new vessel forms (Schnaper *et al*, 1993).

Thrombin, in addition to its involvement in blood coagulation, is mitogenic for capillary, but not large-vessel, endothelial cells, which supports its role in angiogenesis (Griendling & Alexander, 1996). At the site of vessel damage, the activation of platelets and leukocytes, the coagulation cascade and the fibrinolytic system provide positive and negative regulators of angiogenesis. Chronic inflammation can promote angiogenesis whereas angiogenesis can facilitate chronic inflammation. Many previous studies have shown that thrombin stimulates endothelial cell migration and accelerates endothelial cell alignment in Matrigel (Haralabopoulos *et al*, 1997). It also induces angiogenesis in the chick chorioallantoic membrane system (Tsopanoglou *et al*, 1993). Thrombin is a potent stimulator of endothelial cells, releasing of MMP-1, -2, -3 and –9 (Zucker *et al*, 1995; Duhamel-Clerin *et al*, 1997). These MMPs, when released, play an important role in degradation of basement types IV collagen, type I collagen, laminin, and fibronectin during angiogenesis (Unemori *et al*, 1990). MMP-2 and MMP-9 secretion by microvascular endothelium is thought to be responsible for the breakdown of the basement membrane during angiogenesis. Active MMP-9 is stored in intracellular endothelial vesicles ready for release, providing a potentially rapid source of active enzyme. In contrast, proMMP-2 is expressed constitutively by human microvascular endothelial cells (Nguyen *et al*, 1998, Fernandez-Patron *et al*, 1999a). Thrombin is an efficient activator of proMMP-2 by rapidly generating the fully active species. The abundance of thrombin in angiogenic situations such as wound healing, cancer, and arthritis could initiate new blood vessel formation by activating the constitutively expressed proMMP-2 (Zucker *et al*, 1995). Thus, the rapid and efficient activation of proMMP-2 by thrombin in human microvascular endothelial cells represents an effective physiological and pathological mechanism of basement membrane degradation during angiogenesis.

A delicately orchestrated balance between production, activation and inhibition of MMPs is considered to be essential in maintenance of blood vessel integrity. I would like to investigate how platelet-leukocyte-endothelium interactions during thrombin stimulation regulate angiogenesis focusing on the role of MMPs.

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